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# Abscisic Acid And Nitrate Transporter Mtlatd/nip Signaling In Root And Nodule Development In Medicago Truncatula

Chang Zhang

University of Vermont, czhang3@uvm.edu

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ABSCISIC ACID AND NITRATE TRANSPORTER *MTLATD/NIP* SIGNALING IN ROOT  
AND NODULE DEVELOPMENT IN *MEDICAGO TRUNCATULA*

A Dissertation Presented

by

Chang Zhang

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The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements  
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Specializing in Plant Biology

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Dissertation Examination Committee:

Jeanne M. Harris Ph.D., Advisor  
Nicholas Heintz, Ph.D., Chairperson  
Mary Tierney, Ph.D.  
Philip Lintilhac, Ph.D.  
Markus Thali, Ph.D.  
Cynthia J. Forehand, Ph.D., Dean of the Graduate College



## ABSTRACT

Abscissic acid (ABA) is a plant hormone that regulates various developmental processes and environmental stress responses. ABA modulates growth of both primary roots and lateral roots, helping to shape root architecture. The *lateral root organ defective (latd)* mutants, disrupted in the MtLATD/NIP gene, encoding a nitrate transporter, have severe root growth defects that can be rescued by applying ABA. However, the way in which ABA stimulates *latd* root growth is unclear, and the downstream components of MtLATD/NIP and ABA signaling are completely unknown. To answer these questions, this dissertation focuses on two major potential downstream regulators: Reactive Oxygen Species (ROS) and transcription factors (TFs).

ROS are important signaling molecules required in ABA-induced stomatal closure under drought or osmotic stresses, but their role in ABA regulation of root development is unclear. I found that *latd* mutant roots have increased ROS levels, and the expression level of several MtRboh genes, which encode major ROS-producing enzymes, the NADPH oxidases, is also increased. ABA decreases the amount of ROS in *latd* roots and also reduces expression of MtRbohC, in particular. In addition, I observed that *latd* mutant roots have cell elongation defects, which can also be rescued by exogenous ABA. I demonstrated that pharmaceutically decreasing ROS levels using an NADPH oxidase inhibitor, or reducing the expression of MtRbohC using RNA interference can increase cell elongation and stimulate lateral root elongation in *latd* roots. These findings have revealed a mechanism by which ABA restores root growth in *latd* mutant roots via regulating ROS levels, and identified MtRbohC as an important downstream target of ABA signaling mediated by MtLATD/NIP.

TFs act as regulatory nodes controlling the transcription of gene clusters and playing a crucial role in plant growth and development. Using a high-throughput TF profiling approach, I have identified 20 TFs that exhibit altered expression levels in *latd* mutant roots as compared to wild type, 60% of which can be restored to normal levels by ABA. My analysis also revealed that ABA regulates the expression of a different set of TFs in *latd* roots, suggesting that MtLATD/NIP is crucial for ABA regulation of TF expression. Moreover, ABA changes the TFs regulated by MtLATD/NIP almost completely, indicating a tight control of ABA on TFs regulated by MtLATD/NIP. Surprisingly, I found that the expression of *NODULATION SIGNALING PATHWAY 2* (MtNSP2), a GRAS family TF required for nodulation, is regulated by MtLATD/NIP, ABA and nitrate in non-symbiotic roots. In symbiotic roots, MtLATD/NIP is required for the transcriptional signaling pathway downstream of MtNSP2 in the epidermis as well as induction of MtNSP2 expression by cytokinin and subsequent activation of its downstream targets in the cortex. These findings indicate that MtLATD/NIP functions in nodulation signal transduction upstream of MtNSP2, and mediates crosstalk with cytokinin.

Together, these two approaches have begun to characterize a signaling pathway downstream of ABA and MtLATD/NIP that involves ROS, MtNSP2, and a core group of TFs in the regulation of root development and nodulation in *M. truncatula*.

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## **DEDICATION**

This dissertation is dedicated to my mother, my grandfather (lao ye) and grandmother (lao lao).

## **ACKNOWLEDGEMENT**

I would like to express the deepest appreciation to my advisor Dr. Jeanne M. Harris for all the guidance, suggestions, patience, understandings, and encouragements that she offered me throughout these six and a half years. I cannot find better words than that Dr. Harris is simply the greatest mentor to me, who has been holding the light for me and helped me find directions when I was lost, who has led me to a new, different but exciting world of plant biology. She has deeply influenced and inspired me with her enthusiasm and knowledge, and has taught me how to be a logical, deep and independent thinker.

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## **CHAPTER ONE: LITERATURE REVIEW**

## OVERVIEW

This dissertation focuses on the question of how abscisic acid (ABA) and the nitrate transporter MtLATD/NIP regulate root and nodule development in the model legume *Medicago truncatula*. We have taken two different directions in pursuing answers to this major question, by looking at the roles of reactive oxygen species (ROS) during ABA signaling mediated by MtLATD/NIP in root development, and identifying transcription factors that function downstream of the ABA and MtLATD/NIP signaling pathways. Therefore, this review will introduce the current understanding of ABA metabolism and signaling in plants, the function of ROS in plant growth and development and their involvement in ABA signaling, as well as transcription factors known to play major roles in ABA signaling and responses. Since the interest of this dissertation is on how root and nodule development is regulated by ABA and MtLATD/NIP, I will also review the process of root and nodule development, and the major regulators that control these processes. Lastly, I will summarize findings on the function of MtLATD/NIP from all published studies.



## 1.1 ABSCISIC ACID METABOLISM AND SIGNALING

Absciscic acid (ABA) is a plant phytohormone that mediates various responses to environmental signals, such as cold, drought, and salinity. ABA plays important roles in many different developmental stages: it regulates seed dormancy and germination, seedling growth, and stress tolerance for plant survival. ABA is an ancient hormone and is conserved throughout the plant kingdom. It is found in algae, bryophytes, lichens, fungi, and higher plants (Hauser et al., 2011). This review section will be focusing on the findings in ABA biosynthesis and degradation through the key enzymes, as well as ABA perception, signaling transduction and ABA transporters in higher plants.

### 1.1.1 Absciscic Acid Metabolism

ABA is a 15-carbon sesquiterpenoid ( $C_{15}H_{20}O_4$ ) plant hormone. The naturally occurring form is S-(+)-ABA; the side chain of ABA is by definition 2-*cis*, -4-*trans* (Finkelstein, 2013). 2-*Trans* ABA is biologically inactive, but R-(-)-ABA has been shown in several studies to have biological activity (Piotrowska and Bajguz, 2011). Various factors determine the ABA concentration at the site of action, particularly the rate of synthesis, long-distance transport, tissue susceptibility, conjugation, and oxidative degradation (Finkelstein, 2013). This section will provide an overview of the biosynthetic pathway of ABA and its catabolism, as well the regulatory points of the two processes. Then the molecular mechanism of ABA transport will be introduced.

## ABA Biosynthesis

ABA in higher plants is synthesized from an indirect pathway via the cleavage of a C<sub>40</sub> carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA-aldehyde (Culter and Krochko, 1999; Taylor et al., 2000; Seo and Koshiba, 2002; Xiong and Zhu, 2003). This *de novo* biosynthetic pathway starts from the synthesis of the carotenoid precursor in plastids, with the ultimate precursor glyceraldehyde-3-phosphate and pyruvate (Seo and Koshiba, 2002). Chemical inhibitors of carotenoid biosynthesis, such as fluridone and norflurazon, can inhibit ABA accumulation (Gamble and Mullet, 1986). After a C<sub>40</sub> carotenoid precursor has been synthesized, the next step is the specific ABA biosynthetic pathway: xanthophyll formation and its cleavage in plastid. The first step that is more specific to the ABA biosynthesis pathway is the epoxidation of zeaxanthin and antheraxanthin to violaxanthin, which occurs in plastids (Xiong and Zhu, 2003). This step is catalyzed by a zeaxanthin epoxidase (ZEP). After a series of structural modifications, violaxanthin is converted to 9-*cis*-epoxycarotenoid. Oxidative cleavage of the major epoxycarotenoid 9-*cis*-neoxanthin by the 9-*cis*-epoxycarotenoid dioxygenase (NCED) yields a C<sub>15</sub> intermediate, xanthoxin. This step is considered the first committed step in the ABA biosynthesis pathway (Xiong and Zhu, 2003).

The next steps occur in the cytosol. The product xanthoxin, synthesized by NCED in the plastid, is exported to the cytosol, where it is converted to ABA through a two-step reaction via ABA-aldehyde. The conversion from xanthoxin to ABA-aldehyde is catalyzed by a short-chain alcohol dehydrogenase/reductase (SDR), encoded by the *AtABA2* gene (González-Guzmán et al., 2002). The final step, which is the formation of ABA from ABA aldehyde, is catalyzed by ABA aldehyde oxidase (AAO). The molybdenum cofactor (MoCo) synthase encoded by *ABA3* is also required for ABA synthesis at this step, as AAO's cofactor (Bittner et al., 2001; Xiong and Zhu, 2003).

ABA can also be released by the hydrolysis of ABA-GE: active ABA in the cell can be produced directly by hydrolyzing ABA-GE, the biologically inactive, glucose-conjugated form (Lee et al., 2006). ABA-GE is compartmentalized in the vacuole and apoplast (Dietz et al., 2000). Under dehydration stress, bioactive ABA can be released from ABA-GE by a beta-glycosidase homolog, AtBG1, which is localized in the endoplasmic reticulum (ER) (Lee et al., 2006). AtBG1 protein exists as low molecular weight monomers or dimers under normal physiological conditions, but undergoes polymerization induced by dehydration (Lee et al., 2006). In *atbg1* mutants, intracellular ABA levels are comparable to wild-type, but extracellular ABA levels are significantly lower than wild-type (Lee et al., 2006). This indicates that ABA is generated by AtBG1 in the ER then secreted into the apoplast (Lee et al., 2006). AtBG2, which is localized to the vacuole, also has the ability to hydrolyze ABA-GE to release ABA under dehydration and NaCl salt stress (Xu et al., 2012). In *atbg2-2* mutants, ABA levels in the leaf tissue are similar to that of wild-type under normal conditions (Xu et al., 2012), which suggests other regulations for maintaining ABA levels are intact in *atbg2-2* mutants, and that AtBG2 may be functioning in response to specific stresses.

In summary, ABA biosynthesis consists of two pathways: the *de novo* biosynthesis pathway, as well as release from ABA-GE. These two pathways occur at different locations in the cell, but can be compensated by each other, supported by the fact that overexpression of either AtBG2 or AtNCED3 can complement the *atbg1* mutant phenotype (Xu et al., 2012) (Fig. 2).

### **ABA Catabolism**

Two major pathways of ABA catabolism exist: esterification of ABA to ABA-glucosyl ester (ABA-GE), and hydroxylation of ABA at the 8' position by a P-450 type monooxygenase to

give an unstable intermediate, 8'-hydroxy-ABA that rearranges spontaneously to phaseic acid (PA) (Piotrowska and Bajguz, 2011; Finkelstein, 2013) (Fig. 2). The first pathway is reversible; ABA can be released from ABA-GE, while ABA hydroxylation causes irreversible degradation of ABA.

ABA 8'-hydroxylases belong to the Cytochrome P450 CYP707A family (CYP707A1-CYP707A4), with four members in *Arabidopsis* (Kushiro et al., 2004; Saito et al., 2004). Expression of all four CYP707A genes are induced rapidly by drought, and exogenous application of ABA (Kushiro et al., 2004). However, CYP707A3 is shown to exhibit highest expression levels during dehydration and rehydration, which indicates its prominent role in regulating ABA catabolism during dehydration and rehydration (Umezawa et al., 2006). Inhibiting CYP707A chemically using diniconazole will lead to more ABA accumulation in vegetative tissues, and increased drought tolerance (Kitahata et al., 2005). In *cyp7073a* mutants, there are increased levels of ABA under control and dehydration/rehydration conditions, compare to wild-type; phaseic acid levels are decreased under dehydration/rehydration conditions (Umezawa et al., 2006).

Another way to lower ABA levels is that ABA and hydroxyl ABA can be conjugated with glucose and form ABA glucose ester (ABA-GE) by ABA uridine diphosphate glucosyltransferases (UGTs) (Culter and Krochko, 1999; Lee et al., 2006) (Fig. 2). In *Arabidopsis*, the large GT gene family consists of 88 complete sequences (Li et al., 2001). There are 8 GTs that can catalyze the reaction from ABA to ABA-GE in *Arabidopsis* (Finkelstein, 2013). In *Arabidopsis*, AtUGT71B6 was first shown to be important for ABA homeostasis (Priest et al., 2006). AtUGT71B6 and its two close homologs AtUGT71B7 and AtUGT71b8, which encode UGTs that present in the cytosol, can lower the levels of ABA (Dong et al., 2014). Reducing their expression leads to increased ABA levels as well as ABA hypersensitivity for seed germination, seedling growth, and ABA-inducible gene expression (Dong et al., 2014). ABA-GE is

synthesized in the cytosol of root cortex, then released to apoplast and xylem (Sauter et al., 2002) and is recognized as the transport form of ABA (Lee et al., 2006). Considering the plasma membrane as impermeable for ABA-GE, two mechanisms could be involved in the avoidance of extremely high apoplastic concentrations of ABA-GE after it arrives at the leaf: first, the transporters for ABA-GE located in the mesophyll plasmalemma could redistribute the conjugates to the mesophyll cells, and second, apoplastic glucosidases could cleave the physiologically inactive conjugates and release free ABA to their targets, the stomata or the growing cells of young leaves (Sauter et al., 2002).

### **Key Regulatory Points in ABA Metabolism**

ABA biosynthetic enzymes are major regulatory sites of ABA metabolism. In ABA *de novo* biosynthesis, ZEP catalyzes the conversion of zeaxanthin into trans-violaxanthin. AtZEP expression is regulated by cold, salt and dehydration stress, indicating its role in regulating stress responses. ABA also induces its expression rapidly (Xiong et al., 2002). Overexpression of AtZEP resulted in an increased tolerance to salt and drought stress (Park et al., 2008).

Another set of key enzymes in ABA biosynthesis are the NCEDs, which catalyze the production of xanthoxin, the first committed and rate-limiting step. The first identified and cloned NCED gene in plant is the maize *VP14* gene. The maize *vp14* mutant exhibits an ABA-deficient phenotype (Schwartz et al., 1997; Tan et al., 1997). In *Arabidopsis*, there are nine carotenoid cleavage dioxygenase (CCD) genes identified and among them, five NCED genes are closely related to maize *VP14* (Tan et al., 2003). Four of these five NCEDs (AtNCED2, AtNCED3, AtNCED6, AtNCED9) have been directly shown to have 9-*cis* epoxycarotenoid cleavage activity (Iuchi et al., 2001). AtNCED3 expression is induced by drought stress; overexpressing AtNCED3

gene resulted in an increased ABA levels and lower transpiration rates under normal growth conditions (Iuchi et al., 2001). The *AtNCED3* overexpression plants also showed increased drought tolerance under drought stress (Iuchi et al., 2001), suggesting its important role in drought-triggered ABA biosynthesis and responses. Another study using a more sensitive approach detected all *AtNCED* expression was induced by drought stress, although *AtNCED3* has the highest expression levels among the five *AtNCED* genes (Tan et al., 2003). *AtNCED2* and *AtNCED3* are highly root-expressed, while the expression of *AtNCED5* and *AtNCED6* are dominant in flowers (Tan et al., 2003). Different expression pattern of different *AtNCED* genes may contribute to ABA biosynthesis during different developmental stages and functions.

Maize VP14 protein has been shown to localize to plastids (Schwartz et al., 1997), as are the five *Arabidopsis* NCEDs (Tan et al., 2003). Drought stress induces changes of NCED at both gene expression and protein levels. *AtNCED3* protein has two forms: the 64-kD form was mainly located in the thylakoid membrane, while the other form (56 kD) was found in the stroma (Endo et al., 2008). After 1 h of rehydration, levels of both the 64- and 56-kD forms started to decline; after 3 h of rehydration, the 64-kD form was not detectable, while a significant amount of the 56-kD form still remained, which showed that the protein levels of the 64-kD form were correlated with the changing ABA levels (Endo et al., 2008). In dehydrated leaves, *AtNCED3* protein is localized in vascular parenchyma cells, but interestingly not in mesophyll cells, which produce large amount of carotenoids (Endo et al., 2008). *AtNCED3* gene is also expressed only in the vascular tissue of drought stressed leaves (Endo et al., 2008). This suggests the location of ABA biosynthesis induced by drought stress is strictly regulated.

ABA2/GIN1, the short-chain alcohol dehydrogenase, catalyzes the conversion of xanthoxin to abscisic aldehyde. Mutants of this gene were isolated from different genetic screens, which indicates it functions in many developmental processes. The *Arabidopsis* mutant *aba2-1* was first identified from a screen looking for germination in the presence of the GA biosynthesis

inhibitor paclobutrazol (Leon-Kloosterziel et al., 1996). The mutants were found to have lower ABA levels and applying an exogenous ABA analogue can restore their ABA-deficient phenotypes (Leon-Kloosterziel et al., 1996). Biochemical evidence showed that both mutants lack the ability to convert xanthoxin to ABA, while *aba2-1* has normal xanthoxin oxidase activity (Schwartz et al., 1997). Other allele of *ABA2*, *sis4* was isolated from a screen for mutants insensitive to high glucose or sucrose (Laby et al., 2000). *Arabidopsis ABA2* was also identified and cloned from a mutant, *sre-1*, insensitive to NaCl stress, in the aspect of seed germination and early seedling growth (González-Guzmán et al., 2002). Another allele of *aba2-1*, *gin1-3* was isolated from glucose-insensitive screen, indicating the cross-talk between ABA and glucose (Cheng et al., 2002). *ABA2/GIN1* is expressed in various tissue types, with more expression in roots and stem than leaves or seeds (Cheng et al., 2002). Interestingly, in contrast to other ABA biosynthesis genes such as *ZEP1*, *NCED3*, or *AAO3*, the expression of *ABA2/GIN1* is not induced by ABA treatment or dehydration stress (Cheng et al., 2002). Overexpressing *ABA2/GIN1* under the constitutive 35S promoter resulted in more ABA accumulation, delayed seed germination, and increased salt tolerance, but did not promote primary root growth (Lin et al., 2007). A prolonged salinity, cold, flooding, or drought stress treatment can induce *ABA2* promoter activity, although short-term dehydration cannot (Cheng et al., 2002; Lin et al., 2007).

*ABA3/LOS5* encodes a molybdenum cofactor (MoCo) sulfurase, and is required for the activation of aldehyde oxidases (AAO) (Schwartz et al., 1997; Bittner et al., 2001; Xiong et al., 2001). The *ABA3/LOS5* gene expression can be induced by drought, salt or ABA treatment (Bittner et al., 2001; Xiong et al., 2001). Overexpressing *ABA3/LOS5* in tobacco, maize, and soybean can increase drought tolerance (Yue et al., 2012; Li et al., 2013; Lu et al., 2013). AAOs and their cofactor MoCo catalyze last step of ABA biosynthesis: the oxidation of abscisic aldehyde (Taylor et al., 2000). Among the *Arabidopsis AAO* family consisting of four genes *AAO1*, *AAO2*, *AAO3*, and *AAO4*, *AAO3* seems to play a major role since it can efficiently

catalyze the oxidation of abscisic aldehyde to ABA (Seo et al., 2000). The *aao3-1* mutant has a wilted phenotype and reduced ABA content in leaves (Seo et al., 2000). Both drought stress and ABA treatment can induce *AAO3* gene expression rapidly in roots, leaves and guard cells (Seo et al., 2000; Koiwai et al., 2004; Barrero et al., 2006). The AAO protein is localized in the root tips, hypocotyls, and stems; intense signals were detected in phloem companion cells and xylem parenchyma cells, with faint but significant signals observed in guard cells (Koiwai et al., 2004). These results indicate that the ABA is synthesized in the vascular tissue, and that guard cells themselves are able to synthesize ABA (Koiwai et al., 2004). *aao3-2* and *aao3-3* mutants have decreased ABA levels in their seeds (González-Guzmán et al., 2004), suggesting that AAO3 indeed plays a role in ABA synthesis in the seeds.

### **ABA Transport and Transporters**

After ABA is synthesized in one site in the plant, it needs to be transported to the site of action to carry out functions. Based on studies that showed the localization of ABA-biosynthetic enzymes such as NCED3, AAO3 and ABA2, ABA synthesis is thought to happen in vascular tissues, guard cells and seeds (Boursiac et al., 2013). When soil starts to dry, ABA accumulates in the roots, is released to xylem vessels, and transported to leaves through long distance transport for stomatal responses (Sauter et al., 2001). Currently there are two models for ABA transport across cell membrane: the ion-trap model and transport via transporters (Boursiac et al., 2013).

The ion-trap model is based on the observation that ABA is a weak acid with a  $pK_a$  of 4.7, and the uncharged protonated ABA form (ABA-H) can diffuse freely through the cell membrane (Boursiac et al., 2013). In the apoplast where pH is at 5.0 to 6.1, about 50% of ABA is in the ABA-H form; in the cytosol where pH is at about 7.5, very little ABA would be in ABA-H



form. Once ABA synthesized in vasculature reaches guard cells through the flow of xylem sap, ABA-H will be depleted into guard cells by diffusion (Kaiser and Hartung, 1981; Boursiac et al., 2013). However, this model does not explain how ABA flows out of the cell. Also, drought stress leads to an increased pH in the xylem sap, which would make ABA diffusion problematic (Wilkinson and Davies, 1997; Umezawa et al., 2010).

The mechanism of intracellular ABA transport mediated by ABA transporters has only been discovered recently. In *Arabidopsis*, two ATP-BINDING CASSETTE (ABC) transporters, AtABCG25 and AtABCG40, as well as a nitrate transporter AtNPF4.6/NRT1.2/AIT1, are responsible for ABA transport (Kang et al., 2010; Kuromori et al., 2010; Kanno et al., 2012). Both of the two ABCG genes belong to the large ABC transporter family, the majority of which encode membrane-bound proteins transporting a wide range of molecules across the membrane (Verrier et al., 2008). Mutants from these two genes, *abcg25* and *abcg40* respectively, were isolated both as exhibiting altered ABA responses. *ABCG25* is expressed in various tissue types, with higher expression in roots, vasculature and hypocotyls, and its expression is induced by ABA (Kuromori et al., 2010). The ABCG25 protein is localized to plasma membrane, and showed ABA export ability in an ATP-dependent manner using regenerated membrane vesicles from Sf9 insect cells of *Spodoptera frugiperda* (Kuromori et al., 2010). On the other hand, ABCG40 is also plasma-membrane localized and broadly expressed, but it is most highly expressed in the guard cells (Kang et al., 2010). ABCG40 has been shown to have the ability to take up ABA in yeast and in mesophyll protoplasts in a pH-independent way (Kang et al., 2010). Transgenic plants overexpressing ABCG25 are insensitive to ABA for germination and seedling growth, with higher leaf temperature and slower water loss in detached leaves; *abcg25* mutants are hypersensitive to ABA, but do not have any shoot phenotype (Kuromori et al., 2010). It is possible that ABCG25 is an efflux transporter of ABA out of the cell, and ABA may be over-accumulated in the apoplast (cell wall space) of guard cells overexpressing ABCG25 (Kuromori

et al., 2010). ABCG40, on the other hand, is possibly an ABA importer, based on the fact that in *abcg40* mutants, ABA-responsive gene expression is strongly delayed upon ABA treatment, and mutants are impaired in ABA response and drought tolerance, due to reduced stomatal closure (Kang et al., 2010). The fact that both *abcg25* and *abcg40* loss-of-function mutants do not have completely abolished ABA responses, suggests the existence of other ABA intracellular transporters and their functional redundancy. Together, ABCG25 may function in transporting ABA out of the vasculature when ABA arrives at the leaves, either via ABA-synthesizing vascular cells or via long distance transport, then ABCG40 transports the ABA released by ABCG25 into the guard cells, which later causes stomatal closure (Umezawa et al., 2010) (Fig. 3).

ABA IMPORTING TRANSPORTER 1 (AIT1), originally identified as a low-affinity nitrate transporter NPF4.6/NRT1.2 in *Arabidopsis* (Huang et al., 1999), also plays a role in importing ABA into the cell (Kanno et al., 2012). With the presence of 0.1 mM ABA, potential ABA import transporters AIT1 through 4 were isolated from cDNA clones that could induce the interaction between PP2C and ABA receptor using yeast two-hybrid assay (Kanno et al., 2012). AIT1 exhibits ABA import activity in both yeast and insect cells, and it is specific to ABA (Kanno et al., 2012). Loss-of-function *ait1* mutants are less sensitive to ABA and the stem surface has lower temperature probably due to open stomata (Kanno et al., 2012). Although AIT1 is expressed in many different tissue types, it is notably that it is expressed in the vascular tissue in leaves and roots (Kanno et al., 2012), but not in the root tip containing root meristem and elongation zone. Interestingly, nitrate does not compete with ABA as the substrate for NPF4.6/NRT1.2/AIT1, and *npf4.6* mutants do not exhibit altered responses to nitrate, suggesting the biological role of NPF4.6/NRT1.2/AIT1 may not involve mediating ABA and nitrate signaling (Kanno et al., 2013).

AtDTX50, a DTX/Multidrug and Toxic Compound Extrusion (MATE) family member from *Arabidopsis*, has very recently been shown to have ABA efflux transporter activity in *E. coli* and *Xenopus* oocytes cells expressing AtDTX50 heterologously (Zhang et al., 2014). Mesophyll cells of *atdtx50* mutants with preloaded ABA exhibit less ABA release; the mutants are also more sensitive to ABA and more tolerant to drought stress (Zhang et al., 2014). AtDTX50 is expressed in guard cells, with protein subcellular localization on the plasma membrane, suggesting that AtDTX50 is an ABA efflux transporter in the guard cells (Zhang et al., 2014).

In summary, ABCG40 and AIT1/NRT1.2 function in transporting ABA across the plasma membrane into the cell, while ABCG25 and DTX50 are ABA efflux transporters that take ABA out of the vasculature and guard cells, respectively. However, it is highly possible that there are other undiscovered ABA transporters, which may be either redundant to these known transporters, or have different functions in different aspects of ABA-mediated responses, stimulated or activated by different environmental signals.

### **1.1.2 ABA Signaling**

ABA levels accumulate in plants under stress conditions, especially during drought or high salt conditions, and this increase in ABA levels will turn on downstream stress responses and tolerance. During drought stress, ABA promotes stomatal closure to prevent water loss, and turns on the expression of genes that encode products functioning in dehydration tolerance (Nakashima and Yamaguchi-Shinozaki, 2013). Drought and high salt conditions could lead to irreversible loss in crop yields; thus for this reason, understanding ABA signaling is valuable for agricultural applications. This review section will introduce the components in the core ABA

signaling network and their functions, as well as the recent findings in transcriptional reprogramming by ABA.

## **The Core ABA Signaling Network**

### *ABA Perception by PYR/PYL/RCAR Receptors*

The ABA molecule is perceived by ABA receptors (ABAR): PYR/PYL/RCAR. RCARs (as Regulatory Component of ABA Receptors) and PYR/PYL (as Pyrabactin Resistance and PYR1-Like, respectively) were identified in two different studies and published simultaneously (Ma et al., 2009; Park et al., 2009). The RCARs were identified through a yeast two-hybrid assay for ABI1/2 interacting proteins; the PYR/PYLs were isolated from a chemical screen for mutants with altered responses to ABA selective agonist pyrabactin. The PYR/PYL/RCAR family in *Arabidopsis* consists of 14 gene members (*PYR1* and *PYL1-13*, or *RCAR1-14*); this genetic redundancy explains the reason of single mutant screen for ABA receptors being unsuccessful for many years. The PYR/PYL/RCAR family belongs to a START domain superfamily; all family members except for PYL13 are ABA receptors (Fujii et al., 2009; Park et al., 2009). Many of the PYR/PYL/RCAR family members also inhibit PP2Cs phosphatase activity in ABA-dependent manner (Ma et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009).

### *Structural Mechanisms of ABA Recognition and Signaling*

Despite the exciting identification of the ABA receptors, the question of how ABA binds these PYR/PYL/RCAR receptors and how this binding induces downstream signaling remained mystery until the year of 2009. Melcher et al. and Miyazono et al. elucidated how these PYR/PYL/RCAR receptors bind ABA by looking at the crystal structures of the receptors with and without ABA (Melcher et al., 2009; Miyazono et al., 2009). Melcher et al. proposed a “gate-latch-lock” mechanism for ABA receptors: The PYL2 receptors contain an open ligand-binding pocket flanked by a gate-like loop. Upon ABA binding, the gate undergoes conformational changes in two highly conserved  $\beta$ -loops, a swing that closes the ligand entry gate and seals the bound ABA molecule from exposure to the solvent. ABA-induced gate closure creates a new surface that allows the receptor to fit into and competitively inhibit the PP2C active site. A conserved tryptophan in the PP2C inserts directly between the gate and latch, which functions to further lock the receptor in a closed conformation (Melcher et al., 2009). At the same time, Miyazono et al. also provided a similar working model for ABA signaling by looking at another member of the family: PYL1 binds (+)-ABA using the START protein specific ligand-binding site, thereby forming a hydrophobic pocket on the surface of the closed lid. This newly formed interface on (+)-ABA-bound PYL1 tightly interacts with a PP2C domain of ABI1, by using this hydrophobic pocket to cover the active site of ABI1 like a plug (Miyazono et al., 2009). Therefore, the phosphatase activity of ABI1 is inhibited (Miyazono et al., 2009).

Another PYR/PYL/RCAR family member, PYR1, forms a homodimer both with and without the presence of ABA (Nishimura et al., 2009; Santiago et al., 2009). ABA induces the conversion of PYR1 in to a more compact, asymmetric close-lid dimer, by binding one of the subunits of the PYR1 dimers (Nishimura et al., 2009; Santiago et al., 2009). A central cavity is formed by PYR1 Bet v I domain, and ABA can be almost completely buried in this cavity (Santiago et al., 2009). The bound ABA is stabilized by both hydrophobic and polar interactions,

either mediated by water molecules, or by direct interactions between ABA and PYR1 (Santiago et al., 2009).

Point mutations introduced in the two conserved  $\beta$  loops cause a reduction in the capacity of PYR1 to interact with and inhibit the PP2C HAB1 (Park et al., 2009). The mutation does not impair ABA binding, but significantly decreases the stability of the ternary complex ABA–PYR1–PP2C (Santiago et al., 2009). This suggests that the loops surrounding the entry to the ABA binding cavity are required for direct interactions with the phosphatases. Santiago et al. proposed that the interactions between the phosphatase and the lid in its closed conformation stabilize the ternary ABA–receptor–PP2C complex, which could also explain the increased affinity of PYR1 for ABA in the presence of the phosphatase (Santiago et al., 2009).

#### *Structural and Functional Diversity of ABA Receptors*

To date, thirteen PYR/PYL/RCAR family members have been shown to be ABA receptors and ten members have been shown to inhibit PP2Cs phosphatase activity, all but PYL7, PYL11, PYL12, PYL13 (Fujii et al., 2009; Hao et al., 2011). Surprisingly, PYL5, PYL6, PYL8, PYL9, PYL10 have also been shown to inhibit PP2Cs even without the presence of ABA (Hao et al., 2011). It is clear that in the absence of ABA, the oligomerization states of the PYR/PYL/RCAR family members are different: PYR1, PYL1, PYL2 and PYL3 exist as dimers, while PYL4 through PYL10 (except for PYL7) exist as monomers (Santiago et al., 2009; Yin et al., 2009; Szostkiewicz et al., 2010; Dupeux et al., 2011; Hao et al., 2011; Zhang et al., 2012). In the presence of ABA, PYR1 dimers dissociate into monomers, which then have the ability to inhibit PP2Cs (Yin et al., 2009; Dupeux et al., 2011; Hao et al., 2011). Correlating this fact with their abilities in inhibiting PP2Cs activity, it suggests that the dimerization of PYLs prevents the

inhibition of PP2Cs when there is no ABA present (Hao et al., 2011). Therefore, to be able to inhibit PP2Cs activity, being a monomer appears to be necessary (Hao et al., 2011). For example, the apo-PYL3 exists as *cis*- homodimers (Zhang et al., 2012; Zhang et al., 2012) and is able to bind both enantiomers of ABA (Zhang et al., 2013). Upon ABA binding, the apo-PYL3 *cis*- homodimers undergo conformational changes into *trans*- homodimers, and easily dissociate into monomers, which physically interact with PP2Cs (Zhang et al., 2012).

Based on the findings that without the presence of ABA, some of the PYR/PYL/RCAR family members can still inhibit some PP2Cs activity to ensure the basal level of ABA signaling downstream (Miyakawa et al., 2013), it is important for us to understand that ABA signaling needs to happen at a certain level to maintain growth and development. In other words, ABA is indeed a major hormone mediating stress responses, but it is also required for normal development at physiological conditions.

ABA receptors play significant role in ABA signaling and plant survival. Triple and quadruple mutants, *pyr1 pyl1 pyl4* and *pyr1 pyl1 pyl2 pyl4* respectively, display strong ABA insensitivity in seed germination and *RD29A* expression (Park et al., 2009). The pentuple mutants *pyr1 pyl2 pyl4 pyl5 pyl8* and sextuple mutants *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* exhibit reduced seedling growth and seed yield, although they are still viable (Gonzalez-Guzman et al., 2012). However, the ABA response in the pentuple and sextuple mutants is extremely impaired, in aspects of seed germination, root and vegetative growth (Gonzalez-Guzman et al., 2012). These mutants were also more sensitive to drying conditions because of more water loss due to their opened stomata (Gonzalez-Guzman et al., 2012). Transcriptome analysis also showed that the transcriptional regulation by ABA altered dramatically in the sextuple mutants, indicating the central role of ABA receptors in ABA signal transduction (Gonzalez-Guzman et al., 2012). Another *Arabidopsis* mutant containing a point mutation in the gene of *PYL4*, which leads to the formation of a stable complex with ABI3 in the absence of ABA, exhibited enhance ABA

sensitivity in the aspects of seed germination and seedling growth (Pizzio et al., 2013). Overexpressing *PYL4* also showed enhanced ABA-mediated inhibition on seed germination and seedling establishment, as well as drought resistance (Pizzio et al., 2013). *PYL5*, whose expression is down-regulated by ABA, when overexpressed in *Arabidopsis*, conferred hypersensitivity to ABA in seed germination, root growth inhibition and increased drought resistance (Santiago et al., 2009).

All PYR/PYL/RCAR family members (except for PYL13) exhibit the ability to transduce ABA signal and induce downstream gene expression in protoplast (Fujii et al., 2009); however, they are expressed at very different levels according to *Arabidopsis* whole genome arrays (Kilian et al., 2007; Laubinger et al., 2008; Yang et al., 2008). The different members also have different and overlapping expression patterns in both *Arabidopsis* embryos and seedlings (Gonzalez-Guzman et al., 2012), which suggests both functional redundancy and specificity among different family members. It remains unknown if each family member has a distinct role in ABA signaling.

#### *Early Abscissic Acid Signal Transduction*

#### *Protein Phosphatases 2C*

The *Arabidopsis* type 2C protein phosphatase family (PP2C) contains 80 genes across the whole genome (Xue et al., 2008) and can be divided into several subfamilies, containing one clade, PP2C clade A, that plays an important role in ABA signaling (Kerk et al., 2002; Schweighofer et al., 2004). this clade contains ABA-INSENSITIVE 1 (ABI1) And 2 (ABI2), encoding homologous proteins shown to be negative regulators of ABA signaling (Leung et al., 1997; Gosti et al., 1999; Merlot et al., 2001). The dominant mutants *abi1-1* and *abi2-1* exhibit



ABA-insensitive phenotypes, for seed germination and seedling growth (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Meyer et al., 1994; Rodriguez et al., 1998). The recessive mutants of *ABI1* and *ABI2* showed ABA-hypersensitive phenotypes (Gosti et al., 1999; Merlot et al., 2001). Genome-wide transcriptome analysis revealed that the regulation of the majority of ABA-responsive genes is altered in the dominant *abi1-1* mutant (Hoth et al., 2002), indicating the essential role of ABI1 in ABA signal transduction. HAB1 (for HOMOLOGY TO ABI1/ABI2), another clade A PP2C gene homologous to ABI1 and ABI2, has also been shown to be a negative regulator of ABA signaling using both gain-of-function and loss-of-function mutants (Rodriguez et al., 1998; Saez et al., 2004). *ABA-HYPERSENSITIVE GERMINATION3* (*AHG3*), also known as *AtPP2CA*, encodes a PP2C that blocks ABA signal transduction in maize mesophyll protoplasts, and the mutation in this gene causes hypersensitivity to ABA in seed germination (Sheen, 1998; Yoshida et al., 2006). *AHG3* is most active in seeds, and *ahg3* mutants did not show significant hypersensitivity to ABA in other parts of the plants (Yoshida et al., 2006), indicating that different PP2C family members may function in different tissue types or different developmental stages. PP2CA proteins also play roles in vegetative tissue in response to ABA. Double mutant lines of *abi1*, *abi2*, *hab1*, *pp2ca* showed increased sensitivity to ABA-mediated inhibition of seed germination and seedling growth inhibition, as well as water loss (Rubio et al., 2009). Triple mutant lines exhibit extreme hypersensitivity to exogenous ABA in almost all aspects of ABA responses, including seed germination, stomatal movement, seedling growth, and partial constitutive ABA-responsive gene expression in the absence of ABA (Rubio et al., 2009). These observations collected from comparing single, double and triple mutant phenotypes revealed that the role of PP2Cs is negatively regulating ABA signaling, and they may be functionally redundant.

The subcellular localization of the ABI1 protein was shown to be nuclear localized in the *Arabidopsis* protoplast, and the nuclear localization signal (NLS), which is at the end of the

carboxyl-terminal domain, is required for its negative regulatory role of ABA-responsive gene expression (Moes et al., 2008). At the root tip, the ABI1-GFP fusion protein is localized to the lateral root cap and columella cells, but not the root meristem, and showed both cytosolic and nuclear localization (Moes et al., 2008).

The regulation of ABI1 and ABI2 PP2Cs was identified through two separate studies that isolated the *Arabidopsis* PYR/PYL/RCAR ABA receptors (Ma et al., 2009; Park et al., 2009), which were introduced in the previous section. In the presence of ABA, RCAR1/PYL1 binds to ABI1/2 and is able to inhibit its phosphatase activity almost completely (Ma et al., 2009; Park et al., 2009). Using a yeast two-hybrid system, RCAR7/PYL13 was shown to bind to specific PP2Cs, such as ABI1/2, PP2CA, but not to HAB1 (Fuchs et al., 2014).

Besides the PYR/PYL/RCAR ABA receptors that regulate the phosphatase activity of PP2Cs, some transcription factors have also been shown to be regulators of PP2Cs at the level of gene expression. AtMYB20 can directly bind to specific promoter regions of ABI1 and PP2CA and down-regulate their gene expression, which results in enhanced salt stress tolerance (Cui et al., 2013). The homeodomain-leucine zipper (HD-Zip) class I transcription factors AtHB7 and AtHB12, which have been shown to be downstream of PP2Cs in response to ABA, can also bind to the promoter of *ABI1*, *ABI2*, *HAB1*, and *PP2CA*, leading to up-regulation of their gene expression (Valdes et al., 2012).

#### *Protein Kinases in ABA Signaling*

SnRK2 kinases (Sucrose Nonfermenting 1-Related protein Kinases 2) are downstream targets of the PYR/PYL/RCAR-PP2C co-receptors, supported by the fact that triple *pyr1;pyl1;pyl4* and quadruple *pyr1;pyl1;pyl2;pyl4* mutant lines display strong insensitivity to ABA in the aspect of up-regulation of SnRK2 kinase activity (Park et al., 2009). SnRK2 kinases

are present in the cytosol, and can be activated by ABA as fast as 2 min after application in an ABI1-dependent manner (Mustilli et al., 2002; Yoshida et al., 2002). There are 10 genes in the *Arabidopsis* genome that encode SnRK2. The kinase activity of 5 of the SnRK2 proteins can be activated by ABA, and that of 9 can be activated by osmotic and saline stress (Hrabak et al., 2003; Boudsocq et al., 2004). Among these SnRK2s, subclass III, consisting of 3 closely related members, SRK2D/SnRK2.2, SRK2I/SnRK2.3, and SRK2E/OST1/SnRK2.6 (Hrabak et al., 2003), has been most studied. The kinase activity of SnRK2.6 is activated by ABA and osmotic stress, and functions as a positive regulator downstream of ABA signaling (Mustilli et al., 2002; Yoshida et al., 2002; Yoshida et al., 2006). Disruption in the *SnRK2.6* gene leads to insensitivity to ABA-induced stomatal closure, and therefore causes increased water loss in leaves and a wilted phenotype under low humidity conditions. Seed germination and dormancy, however, are not affected, indicating that other SnRKs may be functioning in seeds (Mustilli et al., 2002; Yoshida et al., 2002). The double mutant of *snrk2.2 snrk2.3* exhibits a strong ABA insensitive phenotype for seed germination and dormancy, as well as seedling growth, but with only a minor difference in leaf water loss (Fujii et al., 2007). *SnRK2.6* is expressed specifically in guard cells, while *SnRK2.2* and *SnRK2.3* are expressed in various tissue types, including leaf, root and flower, indicating SnRK2.2 and SnRK2.3 play a role in broader aspects of ABA signaling (Mustilli et al., 2002; Yoshida et al., 2002; Fujii et al., 2007). The triple mutant of *snrk2.2 snrk2.3 snrk2.6* exhibits a much more severe phenotype than the double mutants and is almost completely insensitive to ABA inhibition of germination and early seedling growth, even at high concentrations, such as 50-100  $\mu$ M (Fujii and Zhu, 2009; Nakashima et al., 2009; Umezawa et al., 2009), while the other ABA insensitive mutants usually stop developing at 10  $\mu$ M ABA (Fujii and Zhu, 2009). This triple mutant also shows severe defects in growth and reproduction (Fujii and Zhu, 2009; Nakashima et al., 2009), as well as rapid water loss (Fujii and Zhu, 2009). The ABA-induced kinase activity and ABA-responsive gene expression are both completely abolished

in the triple mutants(Fujii and Zhu, 2009). Thus, these Subclass III SnRK2s are positive regulators required for ABA signaling with functional redundancy.

Many studies have shown that PP2Cs directly regulate SnRK2s activity in early ABA signaling. Group A PP2Cs, including HAB1, ABI1 and ABI2 have been shown to directly bind to subclass III SnRK2s including SnRK2.6, SnRK2.2 and SnRK2.3 *in vivo* and *in planta* (Yoshida et al., 2006; Umezawa et al., 2009; Vlad et al., 2009). In addition, not only SnRK2.6, but also all subclass III SnRK2s are inhibited by *abi1-1* mutation, indicating SnRK2s function downstream of PP2Cs (Mustilli et al., 2002; Yoshida et al., 2006; Umezawa et al., 2009). Furthermore, both *in vitro* and *in vivo* assays showed that in the absence of ABA, PP2Cs directly dephosphorylate subclass III SnRK2s, at the phosphorylation sites in the kinase activation loop, resulting in the inactivation of subclass III SnRK2s (Umezawa et al., 2009). When ABA is present, ABA receptors PYR/PYL/RACRs bind to ABA and PP2Cs, releasing SnRK2s from PP2Cs' inhibition (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009).

Further protein crystal structure analyses revealed more detailed information of the interactions of PP2Cs and subclass III SnRK2s. Analyzing the crystal structures of both SnRK2.6-HAB1 complex and SnRK2.6-ABI1 complex identified at least two interfaces between SnRKs and PP2Cs: one involved a polar interface and the other hydrophobic interface through van der Waals contacts (Soon et al., 2012; Xie et al., 2012). Interestingly, disrupting the hydrophobic interface almost completely abolished the ability of ABI1 to dephosphorylate SnRK2.6, while loss of the polar interface only lead to slight decrease of the affinity of ABI1 for SnRK2.6 (Xie et al., 2012), which puts the hydrophobic interface at a critical position in understanding the mechanism of ABA signaling transduction.

Combining genetic and biochemical data with the crystal structure analysis done on ABA-PYR/PYL/RCAR receptor complex, a working model has been proposed (Fig. 4): in the absence of ABA, PP2Cs inhibit SnRK2s kinase activity by directly contacting the kinase domain.

The kinase domain of SnRK2.6 overlaps with the ABA binding interface of HAB1, and HAB1 structure in HAB1-SnRK2.6 complex is nearly identical to that of the ABA receptor complex HAB1-ABA-PYL2 (Soon et al., 2012). This molecular mimicry of how ABA receptors and SnRKs bind PP2Cs reveals that they both use a similar gate-and-lock mechanism (Soon et al., 2012) (Fig. 4). When ABA is present, PYR/PYL/RCAR receptors recognize and bind to ABA, which leads to significant conformational change of the receptors, giving rise to the PP2C binding site. With the formation of the receptor-ABA-PP2C complex, the active site of PP2C is covered by the receptor, thus inhibiting the phosphatase activity of PP2Cs (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009). After being released from the inhibition of PP2Cs, SnRK2s become active through autophosphorylation, which allows the SnRK2s to relay the ABA signal into several distinct pathways, leading to downstream transcriptional activation, ion channel regulation, and generation of secondary messengers (Ng et al., 2011) (Fig. 5).

Mitogen-activated protein kinases (MAPKs) play important role in protein phosphorylation and signal transduction, and are also involved in ABA and biotic stress responses. The MAPK cascade consists of three protein kinases, MAPKKK (MEK), MAPKK (MKK) and MAPK (MPK), in which sequentially phosphorylation occurs and activates downstream protein kinases (Rodriguez et al., 2010). The kinase activity of some MPKs have been shown to be activated by abiotic stresses such as cold, drought and osmotic stresses, as well as touch and wounding (Ichimura et al., 2000). AtMPK1, AtMPK2 can be phosphorylated and activated by AtMKK3; the expression of all three kinases can be induced by ABA, cold and salt stress (Hwa and Yang, 2008). Overexpressing active AtMKK3 in *Arabidopsis* confers increased salt tolerance and ABA sensitivity (Hwa and Yang, 2008), and this may due to the ability of AtMKK3 to induce *RD29A* and *RD29B* expression (Hua et al., 2006). Two other members of the MPK family, MPK9 and MPK12, expressed mostly highly in guard cells, are activated by ABA during stomatal closure, and are downstream of ABA-induced hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Jammes et al.,

2009). Mutants in these two MPK genes are insensitive to ABA- and H<sub>2</sub>O<sub>2</sub>-induced stomatal closure (Jammes et al., 2009). ABA can also activate AtMEK1 activity, which is required for ABA and stress-induced H<sub>2</sub>O<sub>2</sub> production and CATALASE 1 (CAT1) expression (Xing et al., 2007). *atmek1* mutants are less sensitive to ABA in the aspect of stomatal movement; overexpressing AtMEK1 can enhance drought and salt tolerance, indicating its positive role in ABA signaling (Xing et al., 2007). AtMKK1 and its downstream kinase, AtMPK6, are also involved in ABA-induced H<sub>2</sub>O<sub>2</sub> production, as well as ABA-sugar crosstalk during seed germination (Xing et al., 2008; Xing et al., 2009). Single and double mutants of AtMKK1 and AtMPK6 are insensitive to ABA and glucose in seed germination; the induction of ABA biosynthetic genes, *NCED3* and *ABA2*, by glucose is also abolished in these mutants (Xing et al., 2009). These findings suggest a mechanism in which AtMKK1-AtMPK6 signaling may be a crucial component in ABA-glucose crosstalk. AtMPK6, together with AtMPK3 and AtMPK4, are also regulated by protein phosphatase 2C PP2C5 (Brock et al., 2010). This PP2C5 interacts with the MPKs and negatively regulates their kinase activity, while positively regulating ABA responses in seed germination, stomatal closure and gene expression (Brock et al., 2010). MPK4 and MPK6 are also regulated by another PP2C, AP2C1, but which functions in JA and ethylene signaling (Schweighofer et al., 2007), suggesting their possible function in mediating ABA, JA and ethylene signaling networks. In other species such as rice, maize, and pea, components in the MAPK signaling cascade have also been identified as regulators of ABA and abiotic stress responses. This suggests that the MAPK signaling cascade in ABA signaling is a key regulatory module; however, more details of specificity, interactions and regulations are still waiting to be answered.

Calcium-dependent protein kinases (CDPKs) are also characterized to mediate ABA and abiotic stress responses. CDPKs are one of the protein classes that can bind to calcium (Ca<sup>2+</sup>) through their calmodulin-like domain and regulate downstream targets (Asano et al., 2012).

CDPKs can be found throughout the plant kingdom and protozoans, with 37 genes in *Arabidopsis*, but are absent from yeast and animals (Cheng et al., 2002; Asano et al., 2012). CDPKs have four distinct domains: an N-terminal variable domain, followed by a kinase domain, an autoinhibitory domain and a calmodulin-like domain with four EF-hand  $\text{Ca}^{2+}$ -binding sites (Cheng et al., 2002; Harper and Harmon, 2005). Some CDPKs, such as AtCDPK1 and AtCDPK2, are abiotic stress-inducible, but not responsive to ABA (Urao et al., 1994). AtCDPK4 and its homolog AtCDPK11, can be induced by ABA transiently in the aspect of protein levels, and they both phosphorylate the ABA-responsive bZIP transcription factor ABF1 and ABF4 *in vitro* (Zhu et al., 2007). Disruptions of these two CDPK genes lead to insensitivity to ABA for seed germination, seedling growth, stomatal closure, and induction of ABA-responsive gene expression, as well as insensitivity to salt stress (Zhu et al., 2007), which strongly suggests that CDPK4 and CDPK11 are positive regulators of ABA signaling and ABA-mediated stress responses. AtCDPK12 interacts with and phosphorylates ABI2 *in vitro*, as well as ABF1 and ABF4, two transcription factors functioning in ABA-induced gene expression; the fact that down-regulating AtCDPK12 leads to hypersensitivity to ABA in seed germination and seedling growth suggests that it functions as a negative regulator of ABA signaling (Zhao et al., 2011). AtCDPK32 functions in ABA signaling by regulating gene expression via its binding to ABF4 (Choi et al., 2005). AtCDPK32 phosphorylates ABF4 *in vitro*; overexpressing AtCDPK32 results in increased ABA and salt sensitivity in seed germination (Choi et al., 2005). CDPKs are also involved in ABA-mediated stomatal closure, which is discussed with details in the next section. Overall, CDPKs are important regulators in ABA signaling, and different CDPKs can have different substrates and functions, as well as different interacting partners, in order to mediate different aspects of ABA responses.

### *ABA Signal Transduction Through Ion Channels and Secondary Messengers in Guard Cells*

The modulation of ion channels is also an important step in ABA signal transduction. Guard cells have provided a good single cell model for studying ion channels in ABA signal transduction in stomatal movement. Stomatal pores are formed by two guard cells in the epidermis; they control CO<sub>2</sub> influx for photosynthesis and water loss through plant transpiration, so that they are constantly sensing environmental signals (Kim et al., 2010). Guard cells open or close in response to uptake or loss of solute, mostly potassium (K<sup>+</sup>), chloride (Cl<sup>-</sup>), and malate (Kim et al., 2010). The change of the amount of solutes in guard cells leads to the change in turgor pressure, which eventually leads to stomatal open or close (Pandey et al., 2007). ABA induces stomatal closure and inhibits light-induced stomatal opening, although through different mechanisms (Yin et al., 2013). The simplified model of ABA-induced stomatal closure is: cytosolic calcium (Ca<sup>2+</sup>) concentration is induced by ABA (McAinsh et al., 1992), and this elevated Ca<sup>2+</sup> concentration in the cytosol induces S-type anion channels, causing anion efflux and initial membrane depolarization (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992; Hossy et al., 2003). Then the outward-rectifying potassium (K<sup>+</sup>) channel GORK is activated (Ache et al., 2000; Hossy et al., 2003), leading to further membrane depolarization, and eventually the loss of solutes decreases turgor pressure, cause stomata to close (Raghavendra et al., 2010). In the following section, I will review the role of anion channels, potassium and calcium channels, and secondary messengers including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), nitric oxide (NO), phosphatidic acid (PA) and its regulatory G proteins, in mediating ABA responses in guard cells.

Anion channels can be classified into R-type (R for rapid) and S-type (S for slow), with the latter being a major component involved in membrane depolarization for ABA-induced stomatal closure (Schroeder et al., 2001; Pandey et al., 2007). Both types of anion channels are permeable to a range of anions, such as Cl<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, malate<sup>2-</sup> (Pandey et al., 2007). R-type anion



channels are activated transiently, while S-type anion channels generate slow and sustained anion efflux (Schroeder and Keller, 1992). The *Arabidopsis* guard cell expressed gene *SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1)* encodes a S-type anion channel, with distant homology to fungal and bacterial dicarboxylate/malic acid transport protein (Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009). *SLAC1* is expressed preferentially in guard cells; SLAC1 protein is localized on the plasma membrane, and is essential for stomatal closure in response to ABA (Vahisalu et al., 2008). Studies using the *Xenopus* oocytes heterologous expression system showed that SLAC1 physically interacts with SnRK2.6/OST1, which then phosphorylates it, acting as a positive regulator of ABA signaling (Lee et al., 2009). Members of the Protein Phosphatase 2C family, PP2CA and ABI1, have been shown to interact with and inhibit SLAC1 activity (Geiger et al., 2009; Lee et al., 2009; Brandt et al., 2012). In *Vicia faba*, a similar mechanism has been shown with an *Arabidopsis* SnRK2.6/OST1 homolog, guard cell-specific ABA-activated serine-threonine protein kinase (AAPK), regulating slow anion channels (Li et al., 2000). SLAC1 also interacts with and is activated by two CDPKs, CPK21 and CPK23 (Geiger et al., 2010). The calcium-dependent activation of SLAC1 seems to be regulated by CPK21, since CPK23 exhibits calcium-insensitive activity (Geiger et al., 2010). S-type anion channels are also regulated by CPK3 and CPK6: in *cpk3cpk6* double mutants, the S-type anion channel cannot be activated either by  $\text{Ca}^{2+}$  or ABA; stomatal closure cannot be triggered in response to ABA and  $\text{Ca}^{2+}$  oscillations applied to guard cells (Mori et al., 2006). CPK6 can activate SLAC1 and phosphorylate its N-terminus with higher activity than CPK23, and this activation can be SnRK2.6/OST1-independent (Brandt et al., 2012).

Activation of the anion channels leads to membrane depolarization, which activates the potassium outward-rectifying efflux channel, GUARD CELL OUTWARD RECTIFYING POTASSIUM (GORK) (Ache et al., 2000; Hosy et al., 2003), while inactivating  $\text{K}^{+}$  inward-

rectifying cation channels to prevent  $K^+$  uptake (Li et al., 1998). *GORK* expression in the leaf is regulated by ABA and environmental stresses such as cold, drought and salinity; however, its expression is not regulated by ABA in guard cells (Becker et al., 2003). Superoxide ( $O_2^-$ ), a reactive oxygen molecule giving rise to hydrogen peroxide ( $H_2O_2$ ), which is important in ABA signaling, can also regulate *GORK* transcripts post-transcriptionally through pre-mRNA alternative splicing in program cell death (Wang and Song, 2008; Tran et al., 2013). The  $K^+$  inward channels can be regulated by ABA-and stress-activated kinase SnRK2.6/OST1, which phosphorylates KAT1 to inhibit the channel activity. This indicates the negative regulation of KAT1 is needed for ABA-induced stomatal closure (Sato et al., 2009).

Cytosolic  $Ca^{2+}$  concentrations in the guard cells increase in response to ABA, by either allowing  $Ca^{2+}$  entry from outside of the cell, or by releasing  $Ca^{2+}$  from intracellular stores; in both cases, this  $Ca^{2+}$  influx is via  $Ca^{2+}$  channels (Pandey et al., 2007).  $Ca^{2+}$  channels are activated by ABA and hyperpolarization during ABA signaling and function in regulating cytoplasmic  $Ca^{2+}$  concentration for stomatal closure (Hamilton et al., 2000; Pei et al., 2000). It is predicted that in *Arabidopsis* 41 genes may encode non-selective cation channels permeable to  $Ca^{2+}$ , including the *CYCLIC NUCLEOTIDE GATED CHANNEL (CNGC)* family, and the *GLUTAMATE RECEPTOR (GLR)* family (Pandey et al., 2007). However, most genes encoding functional  $Ca^{2+}$  channel are unknown. Many CNGC family members are activated by cyclic adenosine monophosphate and calmodulin, and are permeable to cations such as  $K^+$  and  $Ca^{2+}$  (Köhler et al., 1999; Leng et al., 1999; Ali et al., 2006). The elevated  $Ca^{2+}$  levels in the cytosol then lead to the inhibition of  $K^+$  inward channels and activation of S- and R-type anion channels, which eventually cause stomatal closure.

$H_2O_2$  is one of the most important secondary messengers in ABA signal transduction in guard cells (Pei et al., 2000; Zhang et al., 2001; Kwak et al., 2003; Jannat et al., 2011). ABA

stimulates H<sub>2</sub>O<sub>2</sub> production in the guard cells in *Arabidopsis* and *Vicia faba*, which can then activate Ca<sup>2+</sup> channels (Pei et al., 2000; Zhang et al., 2001); mutants defective in this process exhibit impaired stomatal closure in response to ABA (Pei et al., 2000). *Arabidopsis* plants carrying mutations in both *RbohD* and *RbohF* genes, which encode O<sub>2</sub><sup>-</sup>-producing NADPH oxidase enzymes, are insensitive to ABA for H<sub>2</sub>O<sub>2</sub> production, Ca<sup>2+</sup> channel activation and stomatal closure, but are able to respond normally to H<sub>2</sub>O<sub>2</sub>, indicating that they function upstream of this signal (Kwak et al., 2003). SnRK2.6/OST1 has been shown to physically interact with and phosphorylate AtRbohF (Sirichandra et al., 2009). The regulation of K<sup>+</sup> and Cl<sup>-</sup> ion channels by H<sub>2</sub>O<sub>2</sub>-mediated Ca<sup>2+</sup> channel activation requires the ABA receptor PYR/PYL/RCAR in guard cells (Wang et al., 2013). Together, these findings have positioned H<sub>2</sub>O<sub>2</sub> generated by NADPH oxidases upstream of Ca<sup>2+</sup> activation, and downstream of PYR/PYL/RACR and SnRK2s in the process of ABA-induced stomatal closure. Moreover, *in vitro* assays showed that the activity of ABI1 and ABI2 is sensitive to H<sub>2</sub>O<sub>2</sub>; high concentrations of H<sub>2</sub>O<sub>2</sub> can inhibit ABI1 and ABI2 activity (Meinhard and Grill, 2001), indicating H<sub>2</sub>O<sub>2</sub> as a secondary messenger of ABA signaling can inhibit the negative regulators in ABA signaling.

In addition to H<sub>2</sub>O<sub>2</sub>, nitric oxide (NO) has also been shown to be an important secondary messenger in ABA-induced stomatal closure. ABA can induce NO production via nitrate reductase (NR), which is required for stomatal closure (Desikan et al., 2002; Neill et al., 2002). Mutants that lack functional NR cannot respond to ABA for NO production and stomatal closure, although NO production can be induced by ABA in *abi1-1* and *abi2-1* mutants, indicating that NO may function upstream of these two phosphatases (Desikan et al., 2002). Interestingly, this ABA-induced NO production in stomata has been shown to be H<sub>2</sub>O<sub>2</sub> dependent, supported by the evidence that H<sub>2</sub>O<sub>2</sub> can also induce NO production, and the NADPH oxidase double mutant *atrbohD/f*, which has reduced superoxide and H<sub>2</sub>O<sub>2</sub> levels (Kwak et al., 2003), exhibits severely

reduced NO production and impaired ABA-induced stomatal closure (Bright et al., 2006). Also, a recent study showed that hydrogen-rich water can also induce NO production and stomatal closure, and this process is again ROS-dependent (Xie et al., 2014).

Phosphatidic acid (PA) is also involved in ABA's regulation of stomatal movement as secondary messengers (Hirayama and Shinozaki, 2007), and it is regulated by *GPA1* (as *G PROTEIN  $\alpha$  SUBUNIT 1*), which is involved in ABA-inhibited stomatal opening. PA levels are induced by ABA, and application of PA induces stomatal closure and inhibits inward rectifying K<sup>+</sup> channel (Jacob et al., 1999). *GPA1* encodes the alpha ( $\alpha$ ) subunit of heterotrimeric G protein, which has been shown play an important role in regulating ion channels in animal cells (Brown and Birnbaumer, 1990; Wickman and Clapham, 1995). The G protein  $\alpha$  subunit ( $G\alpha$ ) binds GTP and then is separated from  $\beta\gamma$  subunit, and certain Phospholipase D (PLD) are regulated by G proteins (Ma et al., 1990; Wang et al., 2001). When PLD $\alpha$ 1 is bound to GPA1<sub>GDP</sub>, its activity is inhibited; GTP binding to GPA1 will release PLD $\alpha$ 1 from GPA1<sub>GTP</sub> and then PLD $\alpha$ 1 becomes active (Zhao and Wang, 2004). In *Arabidopsis*, *GPA1* is expressed in guard cells; the mutation in *GPA1* leads to insensitivity to ABA-inhibited stomatal opening (Wang et al., 2001). As mentioned earlier, ABA inhibits the activity of inward K<sup>+</sup> rectifying channels and activates anion channels (Blatt et al., 1990; Pei et al., 1997); these changes induced by ABA are absent in *gpa1* mutants, which are impaired in ABA-inhibited stomatal opening, although not in ABA-induced stomatal closure (Wang et al., 2001). This means that GPA1 is a critical factor that functions specifically in ABA-inhibited stomatal opening. PA synthesized by PLD $\alpha$ 1 interacts with ABI1 in response to ABA, and this interaction is necessary to remove the inhibitory effect of ABI1 in stomatal closure (Zhang et al., 2004; Mishra et al., 2006). PLD $\alpha$ 1 physically interacts with GDP-bound  $G\alpha$  and this interaction modulates both PLD $\alpha$ 1 and  $G\alpha$  activity (Zhao and Wang, 2004). The *plda1* mutants and *plda1gpa1* double mutants are impaired in both ABA-inhibited stomatal

opening and ABA-induced stomatal closure; introducing a mutation in PLD $\alpha$ 1, perturbing its interaction with G $\alpha$ , results in increased sensitivity to ABA-inhibited stomatal opening (Mishra et al., 2006). Evidence provided in these studies above have separated two different pathways of ABA signaling mediated by PLD $\alpha$ 1 to inhibit stomatal opening and to induce stomatal closure: PLD $\alpha$ 1 interacts with G $\alpha$  to inhibit opening and regulates ABI1 activity through its product PA to stimulate closure (Mishra et al., 2006) (Fig. 6).

The ABA perception mechanism is also different in ABA-induced stomatal closure and ABA-inhibited opening: in the ABA receptor quadruple mutant *pyr1/pyl1/pyl2/pyl4*, ABA-induced closure is impaired, but the inhibition by ABA of stomatal opening is still intact. This suggests that during ABA-inhibited stomatal opening, other unknown ABA receptors must be playing a role in ABA perception (Yin et al., 2013).

#### *ABA Regulates Gene Transcription*

In *Arabidopsis*, ABA regulates the expression of almost 10% of all protein encoding genes; this is a much higher percentage than any other hormone (Fujita et al., 2011). ABA levels increase during various abiotic stresses, such as drought, cold and salinity. Through ABA signal transduction, numerous genes respond to the ABA signal and their gene products are thought to promote stress tolerance (Kreps et al., 2002; Shinozaki et al., 2003). Extensive Microarray analyses have been done looking at gene expression profiles in response to various abiotic stresses, and to exogenous ABA. ABA-regulated genes identified in this way, encode products such as late embryogenesis-abundant (LEA) proteins, dehydration-inducible (RD and ERD) proteins, and cold-inducible proteins, in both *Arabidopsis* and rice (Seki et al., 2002; Seki et al.,

2002; Rabbani et al., 2003). More than half of the drought-inducible genes can also be induced by salt stress and ABA treatment (Seki et al., 2002; Seki et al., 2002). However, some stress-responsive genes cannot be induced by exogenous ABA, indicating an ABA-independent abiotic stress signaling pathway (Zhu, 2002; Nakashima et al., 2009). This review section will introduce the *cis*-acting elements and their binding proteins in the core ABA signaling pathway, as well as the major transcription factors that function in ABA-regulated transcriptional reprogramming.

#### *cis-acting ABRE Elements and Their Binding Proteins, AREB/ABFs*

ABA-responsive elements (ABREs), PyACGTGG/TC, are the major *cis*-acting elements identified to function in ABA-regulated abiotic stress responses, present in the promoters of most ABA-responsive genes and stress-inducible genes (Pla et al., 1993; Ono et al., 1996; Seki et al., 2002). The ABREs belong to G-box family and contain an ACGT-core (Menkens et al., 1995; Shen and Ho, 1995; Fujita et al., 2011). Promoter analyses in ABA-responsive genes showed that two or more ABREs are required for gene expression changes in response to ABA and other specific environmental signals, or the combination of an ABRE with another *cis*-acting element named coupling element (CE) (Uno et al., 2000; Zhang et al., 2005). Three coupling elements have been described: CE1, CE3 and dehydration-responsive elements (DREs) (Yamaguchi-Shinozaki and Shinozaki, 1994; Shen and Ho, 1995; Hobo et al., 1999; Narusaka et al., 2003). The combinations can vary among different plant species: for example, *Arabidopsis* and rice both have ABRE-ABRE pairs, but ABRE-CE3 is present in rice but absent in *Arabidopsis* (Gomez-Porrás et al., 2007). DREs were found in the promoters of many stress-responsive genes such as *RD29A* and *COR15A*, with a 5-bp conserved core CCGAC, or C-repeat, and shown to be involved in both ABA-dependent and ABA-independent stress responses in many species such as

maize, *Arabidopsis*, rice and soybean (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Kizis and Pagès, 2002; Narusaka et al., 2003; Maruyama et al., 2012).

Transcription factors are master regulators that control expression of gene clusters; ABA regulates the expression of transcription factors from many different families, including bZIP, AP2/ERF, MYB, NAC, WRKY, bHLH, described in the following sections (Seki et al., 2002). Many transcription factors achieve this regulation by recognizing and binding to *cis*-acting elements in the promoters of their target genes (Nakashima et al., 2009). ABREs can be recognized by basic leucine zipper (bZIP) family transcription factors named AREB-binding factors (ABFs) (Choi et al., 2000; Kang et al., 2002). There are 75 members in the *Arabidopsis* bZIP transcription factor family, 89 in rice, of those, 13 members belong to the *Arabidopsis* AREB/ABF subfamily (Nakashima et al., 2009; Wei et al., 2012; Marco Llorca et al., 2014). Among them, AREB1/ABF2, AREB2/ABF4 and ABF3 were first identified by yeast one-hybrid assay as ABRE-binding proteins of stress-responsive gene *RD29B* and function in ABA signaling involved in drought and high-salinity tolerance in vegetative tissue (Uno et al., 2000; Kang et al., 2002; Kim et al., 2004; Fujita et al., 2005; Fujita et al., 2011). The triple mutant *areb1 areb2 abf3* has impaired osmotic stress-responsive gene expression, reduced drought tolerance, and is more resistant to ABA in primary root growth but not in seed germination (Yoshida et al., 2010). Overexpressing full length *AREB1/ABF2* is not sufficient to induce changes in stress-responsive gene expression such as *RD29B* without exogenous ABA; but the overexpression lines exhibit earlier *RD29B* induction within 2 hours of ABA application (Fujita et al., 2005). In a separate study, 35S:*ABF2* transgenic plants showed reduced gene expression of *RD29A* and *Sucrose Synthase 1 (SUS1)* in the absence of ABA, but higher induction by a 4-hour NaCl treatment (Kim et al., 2004). Interestingly, overexpressing *AREB1/ABF2A* leads to hypersensitivity to glucose in shoot development, indicating *AREB1/ABF2* also plays a role in glucose signaling (Kim et al.,

2004). A homolog of these three ABFs, ABF1, has lower expression levels in drought, but the quadruple mutants *areb1 areb2 abf3 abf1* exhibit increased sensitivity to drought and decreased sensitivity to ABA in root growth compared to triple mutant *areb1 areb2 abf3*, as well as impaired gene regulation by SnRK2s (Yoshida et al., 2014).

Besides the AREBs/ABFs mentioned above, four other members of the same subfamily, *AtABI5/DPBF1* and its three close homologs, *AtDPBF2*, *EEL/AtDPBF4* and *AREB3/AtDPBF3*, are most strongly expressed in seeds and function in seed development and maturation (Finkelstein and Lynch, 2000; Bensmihen et al., 2005). The *abi5* mutants exhibit insensitivity to ABA in seed germination resembling *abi3* mutants, and later studies have shown that ABI5 interacts with and functions downstream of the B3 domain-containing transcription factor ABI3 (Finkelstein, 1994; Nakamura et al., 2001; Lopez-Molina et al., 2002). The transcription, translation, phosphorylation, degradation and activity of ABI5 are all highly regulated by ABA, with this regulation by ABA only present in a narrow developmental time window: within 2 days after stratification (Lopez-Molina et al., 2001). This short post-germination time period serves as a checkpoint of whether the environment is suitable for seedling growth; experiencing ABA or osmotic stress during this time will lead to growth arrest (Lopez-Molina et al., 2003). Interestingly, ABI5 and its close homologs not only bind to the ACGT core-containing *cis*-elements, the ABREs, but also to other distinct *cis*-elements that do not contain the ACGT core (Kim et al., 2002). These transcription factors bind to the promoters of the *Late Embryogenesis Abundant (LEA)* class genes including *AtEm1* and *AtEm6* and induce their expression during seed maturation (Bensmihen et al., 2002; Carles et al., 2002). ABI5 also works synergistically with ABI4, a transcription factor with the AP2 domain, by binding to downstream genes that share the same binding motifs in their promoters (Finkelstein et al., 1998; Reeves et al., 2011). ABI5 is also regulated at post-translational level. Additionally, yeast two-hybrid assays identified a small



family of ABI5-interacting proteins, named ABI5-binding proteins (ABPs) (Lopez-Molina et al., 2003; Garcia et al., 2008). ABP mRNA and proteins levels accumulate at similar developmental stages and have similar responsiveness to ABA as ABI5, and co-localize in the nuclear bodies with ABI5 (Lopez-Molina et al., 2003). ABP functions as a negative regulator of ABI5 and ABA signaling, by promoting ABI5 degradation via 26S proteasomes: in the presence of ABA, ABP prevents ABI5 from over-accumulation; once the ABA signal is removed, ABP promotes ABI5 degradation in order to release the growth arrest (Lopez-Molina et al., 2001; Lopez-Molina et al., 2003). Other family members of ABPs possibly function at different developmental stages or in response to different signals, and regulate different targets, which leads to both redundancy and complexity in post-transcriptional modification of ABI5 (Garcia et al., 2008).

The AREB/ABF transcription factors localize in the nuclei; there they interact with each other and form hetero- or homo-dimers (Yoshida et al., 2010). AREB/ABF transcription factors also interact with SnRK2 protein kinases, which phosphorylate and activate them (Furihata et al., 2006; Fujii et al., 2007; Sirichandra et al., 2009; Kline et al., 2010; Yoshida et al., 2010). In *snrk2.2/2.3/2.6* triple mutants, ABA-activated phosphorylation of AREB/ABFs is completely lost, and a large percentage of genes down-regulated in response to water stress in *areb1 areb2 abf3* and *snrk2.2/2.3/2.6* triple mutants (about 75%) are overlapping (Fujii and Zhu, 2009; Fujita et al., 2009; Fujita et al., 2013).

In summary, AREBs/ABFs are master regulators that cooperatively induce ABA-responsive gene expression in drought and salt tolerance, and the ABRE/ABF-SnRK2 signal transduction pathway is the major positive regulator of ABA/stress responses in plants (Fujita et al., 2013) (Fig. 7).

### *DREB Transcription Factors in ABA-Mediated Stress Responses*

As mentioned above, ABA also regulates the expression and functioning of transcription factors from many different families. Dehydration-Responsive Elements Binding factors (DREB1/CBFs), which belong to a subfamily of the AP2/ERF transcription factor family, have been identified in many plant species, including *Arabidopsis*, common wheat, rice and soybean, as well as others (Lata and Prasad, 2011). DREBs recognize DREs in the promoters of many stress-responsive genes, such as cold and dehydration-induced *COR78/RD29A* (Yamaguchi-Shinozaki and Shinozaki, 1994). The DREB1/CBF family consists of six genes in *Arabidopsis* and five in rice (Sakuma et al., 2002; Dubouzet et al., 2003). Yeast one-hybrid assays using cDNA libraries prepared from cold- and dehydration-treated *Arabidopsis* plants successfully isolated proteins binding to DREs: AtDREB1A/CBF3 and AtDREB2A, which serve as transcriptional activators that recognize the DRE sequence, but are under different regulation (Liu et al., 1998). The expression of AtDREB1A/CBF3 and its two homologs AtDREB1B/CBF1, AtDREB1C/CBF2 is responsive to low temperature stress, while that of AtDREB2A and its single homolog AtDREB2B is responsive to dehydration and high salt stress (Gilmour et al., 1998; Liu et al., 1998; Nakashima et al., 2000). Three DREB1/CBF family members are thought to be ABA-independent, functionally redundant, while DREB2 family are players functioning in ABA signaling (Medina et al., 1999; Gilmour et al., 2004; Novillo et al., 2004; Novillo et al., 2007). Overexpressing a single *Arabidopsis* DREB1/CBF transcription factor DAtREB1A/CBF3 under the 35S cauliflower mosaic virus promoter resulted in increased cold, dehydration and salt tolerance in *Arabidopsis* (Liu et al., 1998; Kasuga et al., 1999). Overexpression of *Arabidopsis* AtDREB1B/CBF1 resulted in freezing tolerance and up-regulation of COR genes (Jaglo-Ottosen et al., 1998). Transgenic *Arabidopsis* and rice overexpressing OsDREB1A, the orthologs of AtDREB1A from rice, showed increased tolerance to freezing, drought and salt, although these

transgenic *Arabidopsis* plants also exhibited dwarf phenotypes (Dubouzet et al., 2003; Ito et al., 2006). Microarray studies have identified targets of AtDREB1A/CBF3, most of which are drought and cold-inducible genes (Seki et al., 2001). These gene products include many different classes such as LEA proteins, COR/KIN proteins, sugar transport proteins and so on (Seki et al., 2001; Maruyama et al., 2004). On the other hand, the downstream of targets of DREB2A consist many heat-stress (HS) inducible genes, indicating a distinct function of DREB2A in regulating heat stress responses (Sakuma et al., 2006). Overexpressing the constitutively active form of AtDREB2A leads to growth retardation, increased tolerance to drought, freezing and heat stress (Sakuma et al., 2006; Sakuma et al., 2006). Overexpression of AtDREB2C in *Arabidopsis* leads to increased ABA sensitivity, which provides evidence for DREBs functioning in ABA-dependent pathways (Lee et al., 2010). The bZIP transcription factor AREB/ABFs physically interacts with DREB/CBFs *in vivo*, although the function of this interaction is not clear (Lee et al., 2010). Together, DREB TFs are important regulators that mediate responses to various abiotic stresses, with some DREBs directly functioning downstream of ABA signaling.

#### *NAC Family Transcription Factors in ABA Signaling*

The family of plant-specific NAC (NAM/ATAF/CUC) transcription factors also plays important roles in ABA signaling and ABA-mediated stress responses (Nakashima et al., 2014). The large NAC transcription factor family consists of 117 *NAC* genes in *Arabidopsis*, 151 in rice and 152 in soybean and tobacco (Nakashima et al., 2014). The common structural feature of NAC transcription factors is the conserved NAC domain at the N-terminus that consists of 150-160 amino acids (Ooka et al., 2003). NAC transcription factors play roles in biotic stress responses during virus and plant pathogen attack by regulating the expression of defense-related genes

(Wang et al., 2009; Wu et al., 2009; Takasaki et al., 2010; Feng et al., 2014; Liu et al., 2014). Some NAC transcription factors are also regulated by ABA and abiotic stresses.

The expression of ATAF1 is ABA- and drought stress-inducible as well as biotic stress-regulated; the *ataf1* mutants and transgenic plants overexpressing ATAF1 both exhibit increased drought stress-responsive gene expression and drought tolerance, indicating ATAF1 is an important regulator of drought-stress signaling, despite the conflicting observations in two studies (Lu et al., 2007; Wu et al., 2009). Moreover, ATAF1 directly binds to the promoter of ABA biosynthesis gene *NCED3*, leading to the induction of *NCED3* expression and increased ABA levels (Jensen et al., 2013). This suggests the NAC transcription factor ATAF1 is also a positive regulator of ABA biosynthesis. Interestingly, ATAF1 itself contains ABRE sequences in its promoter, where the AREB/ABF bZIP transcription factors can recognize and bind (Lu et al., 2007), suggesting NAC transcription factors may be regulated by some bZIP transcription factors and they may function together as a complex to regulate downstream ABA-responsive gene expression. Recently, direct evidence of this interaction has been revealed: *Arabidopsis* ANAC096 interacts with ABF2 both *in vitro* and *in vivo*, to turn on *RD29A* expression and to improve drought stress tolerance (Xu et al., 2013).

Several other NAC transcription factors are also involved in ABA-dependent abiotic stress responses. The expression of NAC transcription factor *AtRD26* is highly induced by ABA, drought and high salinity; transgenic *Arabidopsis* plants overexpressing *RD26* exhibit hypersensitivity to ABA in the aspect of seedling growth (Fujita et al., 2004). Many ABA and abiotic stress responsive genes are mis-regulated in *AtRD26* overexpression and repression lines, together with some defense-related genes and ROS-detoxification genes, although the direct downstream targets of *AtRD26* are still unclear (Fujita et al., 2004). *Arabidopsis* *AtANAC019*, *AtANAC055*, and *AtANAC072*, which were identified initially as binding factors to the promoter

of *Early Responsive to Dehydration stress 1 (ERD1)*, are also induced by ABA, drought, and salt stress at transcriptional level (Tran et al., 2004). Overexpressing these NACs can increase drought tolerance, and AtANAC019 and AtANAC055 are also shown to be a regulator of jasmonic acid (JA) signaling by directly binding to JA-induced genes (Tran et al., 2004; Bu et al., 2008), suggesting these NAC transcription factors may be in the regulatory node of JA and ABA signaling networks. AtNAC1 and AtNAC2 function in the regulation of lateral root development: AtNAC1 activates auxin-responsive gene expression through TIR1-dependent pathway; AtNAC2 integrates environmental and hormonal signaling in lateral root development (Xie et al., 2000; He et al., 2005). AtNAC2 expression is induced by ABA and high salt, with the latter requiring functional ethylene and auxin signaling (He et al., 2005). In rice, OsSNAC1 is an ABA-, cold- and salinity-induced NAC transcription factor, and overexpressing OsSNAC1 transgenic rice can increase drought and salt tolerance (Hu et al., 2006). OsNAC5 plays a role in leaf senescence by targeting chlorophyll degradation-related genes and other senescence-related genes, and is exclusively responsive to ABA but not other hormones or stress signals (Sperotto et al., 2009; Takasaki et al., 2010; Liang et al., 2014). A maize NAC transcription factor ZmSNAC1 acts as a positive regulator of abiotic stress tolerance, and can be phosphorylated by the SnRK2 ZmOST1 (Lu et al., 2012; Vilela et al., 2013), which provided more evidence of NAC transcription factors being an important regulatory component in ABA signaling.

#### *MYB Transcription Factors in ABA Signaling*

MYB transcription factors are a large and diverse family present in all eukaryotes; they regulate plant growth and development, as well as abiotic and biotic stress responses (Dubos et al., 2010). MYB transcription factors contain the MYB DNA-binding domain, which consists of up

to four imperfect amino acid sequence repeats containing tryptophan residues (Dubos et al., 2010). Many MYB transcription factors are of the R2R3-type, based on the number of adjacent repeats, and many R2R3-MYBs are important regulators of plant-specific processes (Martin and Paz-Ares, 1997; Dubos et al., 2010). There are over 100 R2R3-MYBs in *Arabidopsis*, and they are involved in multiple cellular and developmental processes: they regulate secondary metabolism, cell fate and cell cycle, meristem formation, and responses to environmental signals (Jin and Martin, 1999). The first MYB transcription factor responsive to ABA and abiotic stresses was AtMYB2 (Urao et al., 1993). Later studies have shown that AtMYB2 recognizes and binds to the promoter region of a dehydration-responsive gene *RD22*, and overexpressing *AtMYB2* leads to ABA hypersensitivity and up-regulation of many ABA-inducible genes (Abe et al., 1997; Abe et al., 2003). Other members of the R2R3-MYB subfamily have also been extensively studied and shown to be ABA-responsive and regulate abiotic stress tolerance in *Arabidopsis*, rice, wheat, tobacco and other plant species (Dai et al., 2007; Jung et al., 2008; Ding et al., 2009; Yang et al., 2012; Zhang et al., 2012; Huang et al., 2013; Al-Attala et al., 2014; Chen et al., 2014; Su et al., 2014; Wang et al., 2014).

The mechanisms of how several MYB transcription factors function in ABA signaling and abiotic stress responses have been reported from different studies in *Arabidopsis*. AtMYB20 down-regulates the expression of *PP2Cs*, which act as negative regulators in ABA signaling, in order to enhance salt tolerance (Cui et al., 2013). AtMYB96 is also ABA- and drought-inducible at transcriptional levels, but auxin can also induce its expression, mostly in roots (Seo et al., 2009). AtMYB96 incorporates auxin signaling into ABA and drought stress responses by regulating the expression of *GH3* genes, which encode a subset of IAA-conjugating enzymes (Seo et al., 2009). As a result of negatively modulating auxin levels in roots, AtMYB96 inhibits lateral root development under drought conditions (Seo et al., 2009). AtMYB33 and AtMYB101

act as positive regulators of ABA signaling during seed germination and are regulated by an ABA-inducible microRNA, miR159, in an ABI3-dependent manner (Reyes and Chua, 2007). Interestingly, several MYB transcription factors interact directly with the ABA receptor. AtMYB44 was identified as an interacting protein with the cytoplasm and nuclear ABA receptor RACR1/PYL9: this allows AtMYB44 to compete with ABI1 for binding the ABA receptor *in vitro*, and may reduce the inhibitory effect of RCAR1/PYL9 on ABI1's phosphatase activity in the presence of ABA *in vivo* (Li et al., 2014). A different member of the PYR/PYL/RCAR ABA receptor family, PYL8, directly interacts with MYB77, MYB44 and MYB73, then promotes the binding of MYB73 to multiple auxin-responsive genes, which leads to the recovery of lateral root growth inhibition caused by ABA (Zhao et al., 2014). AtMYB7, another member of the R2R3 subfamily which is expressed in mature seeds, negatively regulates the expression of *ABI5*, the bZIP transcription factor that plays central role in ABA-regulated seed maturation (Kim et al., 2014). Thus, several MYB transcription factors are central to ABA signaling, responding to ABA receptor activation itself.

#### *WRKY Transcription Factors in ABA Signaling*

WRKY transcription factors were first studied as regulators of plant responses to pathogens; only recently has their role in stress responses, especially those mediated by ABA, been revealed (Rushton et al., 2010). These transcription factors contain the DNA-binding WRKY domain, consisting of the highly conserved motif WRKYGQK at the N-terminus, and a zinc finger structure at the C-terminus (Rushton et al., 1996; Rushton et al., 2012; Marco Llorca et al., 2014). The W-box, either single or clustered, is the minimal consensus sequence required for WRKY transcription factor binding, with the sequences outside of the W-box determining

binding specificity (Yamasaki et al., 2005; Ciolkowski et al., 2008; Rushton et al., 2010). Many WRKY transcription factors play roles in ABA signaling by interacting with important ABA-signaling components. Mutation in the *AtWRKY63/ABO3* gene resulted in ABA-hypersensitivity for seedling growth, decreased drought tolerance and impaired stress-responsive gene induction upon ABA treatment (Ren et al., 2010). *AtWRKY63* may recognize and bind to the W-box present in the bZIP transcription factor *ABF2* (Ren et al., 2010). *AtWRKY18* and *AtWRKY60* function as transcriptional activators in ABA sensitivity and response to salt and osmotic stresses; *AtWRKY18* and *AtWRKY60* may possibly bind to the W-box of another WRKY transcription factor, *AtWRKY40*, a negative regulator in ABA signaling (Chen et al., 2010). Together, these three WRKY transcription factors directly target the promoter of *ABI4* and *ABI5* to repress their expression, but may act in different mechanisms (Liu et al., 2012). *AtWRKY40* is shown to have central role in negatively regulating ABA signaling, by interacting with the chloroplast-localized ABA receptor *ABAR* and binding to the promoter region of many essential ABA-signaling and responsive genes *in vivo*: *ABI4*, *ABI5*, *ABF4*, *MYB2*, *DREB1A*, *DREB2A*, and *RAB18* (Shang et al., 2010). ABA also induces the expression of *AtWRKY2* during seed germination and postgermination growth arrest in an *ABI3*- and *ABI5*-dependent manner; disruption of *AtWRKY2* gene leads to ABA-hypersensitivity (Jiang and Yu, 2009). Constitutive expression of *AtWRKY57* can increase ABA levels and thus increase drought tolerance, by directly binding to the W-box of the promoter of ABA-biosynthetic genes, *NCED3* and *ABA3*, as well as ABA-responsive gene, *RD29A* (Jiang et al., 2012). The activity of WRKY transcription factors can be controlled by phosphorylation mediated by a MAPK signaling cascade during immune responses (Ishihama et al., 2011; Mao et al., 2011; Shen et al., 2012), but whether the same post-translational regulation exists in ABA signaling and abiotic stress responses is still unknown.



### *bHLH Transcription Factors in ABA Responses*

bHLH TFs play major roles in plant growth and development, and several function in the ABA response pathway. AtMYC2, a basic-helix-loop-helix (bHLH) transcription factor, was isolated together with AtMYB2 as binding factors of the promoter of the drought-responsive gene, RD22 (Abe et al., 1997). Many aspects of AtMYC2's function in ABA signaling are comparable to AtMYB2: they both serve as transcriptional activators in ABA-inducible genes under drought stress conditions (Abe et al., 2003). AtMYC2 also plays an important role in JA signaling, and in the cross-talk between JA and ABA signaling pathways (Kazan and Manners, 2013). AtMYC2 acts upstream of two NAC transcription factors, ANAC019 and ANAC055, both in ABA and JA signaling, based on the fact that in *atmyc2* mutants these two NAC genes cannot be induced by either ABA or JA (Jiang et al., 2009). AtAIG1, another bHLH transcription factor, has shown to be a regulator in ABA signaling; plants lacking functional AtAIG1 exhibit increased ABA sensitivity and reduced ABA-responsive gene induction (Kim and Kim, 2006). ABA also induced the expression of AtAIB bHLH transcription factor; overexpressing AtAIB leads to increased ABA sensitivity in seedling growth and drought tolerance (Li et al., 2007). During ABA-inhibited stomatal opening, the phosphorylation of three bHLH transcription factors, AKS1, AKS2 and AKS3, is promoted by ABA in the guard cells (Takahashi et al., 2013). AKS1 binds directly to the promoter of KAT1, which encodes an inward rectifying K<sup>+</sup> channel, and the phosphorylation of AKS1 by ABA negatively affects this binding, thus suppresses the activity of the K<sup>+</sup> channel and stomatal opening (Takahashi et al., 2013). AtbHLH122 expression is drought- and salt-stress inducible, but its expression does not seem to respond to ABA treatment. However, bHLH122 can bind to the promoter of CYP707A3, a gene encoding a major ABA 8'-hydroxylase for ABA

catabolism, and repress its expression (Liu et al., 2014). Thus bHLH TFs play a key role in ABA signaling at several points in stress responses and regulation of stomatal aperture.

#### *GRAS TFs in ABA signaling*

Some DELLA proteins and SCARECROW-LIKE (SCL) proteins, which belong to the GRAS transcription factor family, are also regulated by ABA, and have important functions in the ABA and gibberellic acid (GA) crosstalk during seed germination, where these two hormones have antagonistic effects (Golldack et al., 2013). Seed germination is correlated with high GA levels and low ABA levels. One of the four DELLA proteins, RGL2, binds to the promoter of XERICO and up-regulates its expression. XERICO is a RING-H2-type zinc-finger protein that contributes to the increase of ABA levels and increased drought tolerance (Ko et al., 2006; Zentella et al., 2007). ABA can induce *RGL2* expression in seeds, which is also induced by inhibiting GA biosynthesis (Piskurewicz et al., 2008). When GA levels are low, RGL2 is required for the induction of *ABI5* expression and the increase of ABA levels (Piskurewicz et al., 2008), which leads to inhibition of germination. Thus the RGL2 TF is important in ABA signaling in the seed, but little is known about the interaction between DELLAs and ABA signaling in root growth.

Recently, a study revealed that *Arabidopsis* SCARECROW (SCR), a GRAS family TF that is essential for root meristem patterning (Di Laurenzio et al., 1996; Sabatini et al., 2003), directly represses *ABA INSENSITIVE 4* and *5* (*ABI4* and *ABI5*) expression in root apical meristem; expressing *ABI4* under *SCR* promoter resulted in a short-root phenotype (Cui et al., 2012). *scr* mutants are also hypersensitive to ABA (Cui et al., 2012), suggesting a negative role of SCR in mediating ABA responses. These results indicated a direct link between GRAS family TFs and

ABA signaling in regulating root development, with GRAS TFs possibly function as repressors that target the core ABA signaling TFs.

### **1.1.3 Evolution of ABA Metabolism and Signaling**

ABA is a small molecule involved with stress responses that is present in all kingdoms of life except for Archaea (Hauser et al., 2011). ABA is detected not only in higher plants, but also in cyanobacteria, sponges, algae, bryophytes, fungi and lichens and even in human granulocytes (Zocchi et al., 2003; Bruzzone et al., 2007; Hartung, 2010; Hauser et al., 2011). Based on sequence comparisons, the core ABA signaling components are only present in land plants, consistent with the fact that ABA is involved with dehydration stress and may contribute to land colonization (Hauser et al., 2011; Miyakawa et al., 2013).

Notably, ABA receptors of the PYR/PYL/RCAR family and downstream transcriptional regulators first occurred in land plants, suggesting that the emergence of core ABA signaling may have evolved as a result of shortage of water in non-aqueous habitats (Hauser et al., 2011; Miyakawa et al., 2013). Comparing protein structures of 149 PYR/PYL/RCAR homologs from 23 plant species showed high conservation in ABA and PP2C binding residues, as well as the catalytic center (Hauser et al., 2011). The earliest emergence of AtPYR/PYL/RCAR homologs is found in the liverwort *Marchantia polymorpha*, which is at the basal lineage of the land plant phylum (Hauser et al., 2011). The most ancient PP2C with a characterized function in ABA signaling, *MpABII*, an ortholog of *Arabidopsis ABII*, functions as a negative regulator in ABA signaling; mutants of *MpABII* showed reduced freezing and osmotic stress tolerance (Tougane et al., 2010). In the moss, *Physcomitrella patens*, there are two *Arabidopsis ABII*-related genes, and their expression is induced by ABA, just as in *Arabidopsis* (Komatsu et al., 2009). *PpABII* also

functions as a negative regulator of ABA signaling; transient expression of *PpABII* blocks the induction of *Em* gene expression by ABA and disruption of this gene leads to ABA hypersensitivity and altered stress tolerance (Komatsu et al., 2009). These results provided evidence that the PP2Cs in ABA signaling are functionally conserved between angiosperms and bryophytes (Komatsu et al., 2009). Protein kinases that function in ABA signaling have also been identified in many different plant species. SnRK2s can be divided into three subclasses with each subclass highly conserved in higher plants (Umezawa et al., 2010). SnRK2s have also been identified in algae and mosses, but those in algae lack the PP2C-binding domain, suggesting that they may be functionally distinct (Hauser et al., 2011). *Arabidopsis* membrane proteins such as voltage-dependent anion channels in ABA-regulated stomatal movements, have close homologs in monocots, as well as the unicellular green algae *Chlamydomonas* (Hauser et al., 2011). Transcription factor families that function in ABA signaling are found in land plants, algae and other eukaryotes, but bZIP AREB/ABF transcription factors are specific to land plants.

ABA metabolism pathways that involves NCEDs catalyzing the rate-limiting step in ABA biosynthesis, seems to have evolved during land colonization, because of the evidence that the most ancient homolog found is in *Physcomitrella*. On the other hand, ABA catabolism enzymes, members of the cytochrome P450 family catalyzing the degradation of ABA, are absent in algae and moss, indicating that degradation of ABA may have evolved later, after colonization of land (Mizutani and Ohta, 2010).

## **1.2 REACTIVE OXYGEN SPECIES (ROS)**

ROS are important signaling molecules that play roles in many aspects of plant development and defense responses, regulating plant growth with biotic and abiotic stress signals. First recognized as toxic molecules, ROS can cause oxidative damage to the cell; as a result, the control of ROS homeostasis is critical. This review section will focus on ROS generation and signaling, as well as the regulation of ROS homeostasis.

### **1.2.1 ROS Production and Scavenging in Plants**

ROS are partially reduced products from oxygen ( $O_2$ ), and exist as superoxide ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $^1O_2$ ), and hydroxyl radicals ( $OH^{\cdot}$ ). Under normal conditions, because of the toxic and highly reactive properties of ROS molecules, plants evolved ROS-scavenging systems to keep ROS levels low. However, during stress conditions, ROS levels can increase dramatically and trigger downstream signaling.

#### **The Production of ROS**

In plant cells, organelles with highly oxidizing metabolic activity are major sources of ROS production, such as mitochondria and chloroplasts, are major sources of ROS production during the processes of respiration and photosynthesis, respectively (Mittler et al., 2004). ROS are also produced in peroxisomes, cytoplasm, ER and cell walls (Mittler et al., 2004; Schmitt et al., 2014).

ROS are produced as unavoidable by-products of essential metabolic processes in the cell by a network of ROS-producing enzymes (Mittler et al., 2004; Suzuki et al., 2011). The plant

Respiratory Burst Oxidase Homologues (Rbohs), or NADPH oxidases, are key enzymes in ROS signaling (Fig. 8). The *Rboh* gene family in *Arabidopsis* consists of ten members, some of which have close homology with the mammalian respiratory burst oxidase catalytic subunit gp91<sup>phox</sup> (phox for phagocytes) of NOX2 (Torres et al., 1998; Suzuki et al., 2011). The mammalian NOX family members all contain transmembrane domains and transport electrons across the cell membrane to reduce O<sub>2</sub> to O<sub>2</sub><sup>-</sup> (Bedard and Krause, 2007). They share conserved structural features: there is an NADPH-binding site at the extreme C-terminus, a FAD-binding region, six transmembrane domains and four highly conserved heme-binding histidines (Bedard and Krause, 2007). In plants, all Rboh proteins share structural homology at the C-terminus with mammalian NOX family members, but they contain a distinct N-terminal region with two Ca<sup>2+</sup>-binding EF-hands, which is absent from gp91<sup>phox</sup>, but present in other mammalian NADPH oxidases such as NOX5 and Duox (Keller et al., 1998; Oda et al., 2010; Suzuki et al., 2011). Plant Rbohs catalyze the reaction from O<sub>2</sub> to O<sub>2</sub><sup>-</sup>, and this reaction can be blocked by chemicals such as diphenylene iodonium (DPI), which is an inhibitor of NADPH oxidases (Hancock and Jones, 1987). These Rbohs localize on the plasma membrane and thus are thought to be responsible for O<sub>2</sub><sup>-</sup> generation in the cell wall.

ROS can also be generated by other enzymes: H<sub>2</sub>O<sub>2</sub> can be generated by superoxide dismutases (SODs) from O<sub>2</sub><sup>-</sup>. SODs are also recognized as part of the ROS-scavenging system because of its ability to scavenge O<sub>2</sub><sup>-</sup>. SODs take one molecule of O<sub>2</sub><sup>-</sup> and reduce it into H<sub>2</sub>O<sub>2</sub>, and another O<sub>2</sub><sup>-</sup> molecule and convert it into O<sub>2</sub> (Halliwell, 2006). SODs can be found at all cellular locations where O<sub>2</sub><sup>-</sup> is produced, likely the reason that O<sub>2</sub><sup>-</sup> is found to be short-lived with half-life in 10<sup>-6</sup> seconds so that it is rapidly converted to H<sub>2</sub>O<sub>2</sub> by SODs (Schmitt et al., 2014). SODs can be classified into three groups: iron SOD (FeSOD), copper/zinc SOD (Cu/ZnSOD) and manganese SOD (MnSOD). FeSODs are localized in the chloroplast, MnSODs in the mitochondrion and peroxisomes, Cu/ZnSOD in the cytoplasm and extracellular space (Alscher et

al., 2002).  $\text{H}_2\text{O}_2$  has a relatively long life, compared to other ROS molecules, with a half-life time of about 1 millisecond. As a result, it can pass through the cell membrane either freely or via aquaporins and move within the cell between different compartments to trigger downstream signaling (Bienert et al., 2007; Quan et al., 2008).

Hydroxyl radicals ( $\text{OH}\cdot$ ), is by far the most reactive type of ROS molecule; it can be generated by the iron-catalyzed Harber-Weiss cycle *in vivo* from  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  (Kehrer, 2000).  $\text{OH}\cdot$  can attack protein and lipids instantaneously at the site of production, which leads to membrane protein and lipids damage and further membrane integrity loss (Halliwell, 2006). The plant specific Class III peroxidases, which are cell wall bounded specifically, can also produce  $\text{H}_2\text{O}_2$  or  $\text{OH}\cdot$  (Francoz et al., 2014).

### **The Scavenging of ROS**

Since ROS are toxic and can cause severe damage to the cell, but on the other hand are by-products of many crucial biological processes, plant cells acquired two mechanisms to maintain a low level of ROS: one of controlling the production of ROS to a very low level; the other one of quickly scavenging excess ROS to avoid cellular damage (Mittler et al., 2004; Halliwell, 2006). Stress conditions, for example, drought and high light conditions can increase the production of ROS; plants may use anatomical adjustments such as leaf movement and curling, or molecular mechanisms that rearrange the photosynthetic apparatus and antenna complex to balance the amount of light being harvested (Mittler et al., 2004). Another way to decrease the production of ROS in mitochondria and chloroplast is via alternative oxidase (AOX): AOX can divert electrons that flow through the ETC and use them to reduce  $\text{O}_2$  to  $\text{H}_2\text{O}$  (Maxwell

et al., 1999; Mittler et al., 2004; Fu et al., 2012). In this way, AOX not only prevent electron from reducing  $O_2$  to  $O_2^-$ , but also reduce the overall  $O_2$  level in these organelles (Mittler, 2002).

Major ROS-scavenging enzymes include SOD, Ascorbate Peroxidase (APX), Glutathione Peroxidase (GPX) and Catalase (CAT). The activities of these enzymes maintain  $O_2^-$  and  $H_2O_2$  levels low, and prevent the production of  $HO^\cdot$ . CAT catalyze the dismutation of two  $H_2O_2$  molecules into  $H_2O$  and  $O_2$ ; the CAT family in *Arabidopsis* contains three genes and they are thought to be localized to peroxisomes, although this is not completely clear yet (Frugoli et al., 1996; Mhamdi et al., 2010). APX and GPX function in the Ascorbate (AsA)-Glutathione (GSH) cycle for the scavenging of  $H_2O_2$ , which either uses electrons from photosynthesis directly or from NADPH as reducing power (Mittler, 2002). Different ROS-scavenging enzymes are localized in different cellular compartments; the ROS-scavenging network can be found in almost all subcellular locations, although different enzymes might have different activities. For example, APX and CAT have higher activity in peroxisomes, while GPX and some APX are more active in the cytosol (Mittler et al., 2004).

### 1.2.2 ROS Functions and Signaling

Despite the toxic nature of ROS molecules, ROS are also important signaling molecules that regulate various biological processes, including triggering immune responses under pathogen attack and mediating abiotic stress responses. They are also crucial for normal plant growth and development. So far, whether ROS signal through certain receptors or sensors is unknown. However, many studies have revealed that ROS signaling requires  $Ca^{2+}$  and phosphorylation by protein kinases. This section will focus on ROS function and signaling via key regulatory



enzymes, and role of ROS as secondary messenger in ABA signaling, as well as in legume-rhizobial symbiosis.

### **ROS Signaling via Plant Rboh/NADPH oxidases**

As mentioned in the section above, plant Rboh/NADPH oxidases are major enzymes producing  $O_2^{\cdot-}$ , which give rise to other forms of ROS molecules; as a result, plant Rbohs are key components in ROS signaling network and play important role in regulating plant development and signaling responses. Under normal conditions,  $O_2^{\cdot-}$  is localized to the root meristem and elongation zone of an *Arabidopsis* root tip (Dunand et al., 2007; Dunand and Penel, 2007). Genes encoding Rboh enzymes have been identified in many plant species, such as *Arabidopsis*, rice, *Medicago truncatula*, etc. *Arabidopsis RbohC/RHD2*, which encodes a plant NADPH oxidase, is required for normal root hair tip growth through the interaction with  $Ca^{2+}$  channels (Foreman et al., 2003; Takeda et al., 2008). *In situ* hybridization detected *AtRbohC* transcript in epidermal cells of the root tip (Foreman et al., 2003); the AtRbohC protein is localized on the plasma membrane of the growing root hair (Takeda et al., 2008). AtRbohC is essential for  $O_2^{\cdot-}$  accumulation at the growing root tip; without a functional AtRbohC leads to defects in root hair and root growth (Foreman et al., 2003). AtRbohC has also been shown to regulate the sensing of mechanical stimulation (Monshausen et al., 2009).

*Arabidopsis RbohD* and *RbohF* are required for ROS accumulation in defense responses during bacterial pathogen attack; mutants of these two genes have less ROS accumulation and reduced cell death (Torres et al., 2002). Because plant Rbohs are localized to the plasma membrane, they are considered to be major contributors to extracellular ROS generation, which can affect cell wall function and therefore growth. AtRbohD is also involved in lignin deposition

in the elongation zone of *Arabidopsis* growing roots during plant-pathogen interactions (Hamann et al., 2009; Denness et al., 2011). AtRbohD and AtRbohF are also important for ABA signaling in guard cells: *atrbohdf* double mutants exhibit impaired ABA-induced responses, including stomatal closure, ROS production, and  $\text{Ca}^{2+}$  channel activation (Kwak et al., 2003). The *atrbohdf* double mutants are also insensitive to ABA-inhibited seed germination and root growth, indicating that AtRbohD and AtRbohF play a much broader role in regulating many ABA responses (Kwak et al., 2003). Later, AtRbohD and AtRbohF were shown to regulate auxin sensitivity on roots grown on ABA, suggesting a possible mechanism of how ABA inhibits root growth in *Arabidopsis* (Jiao et al., 2013). Moreover, AtRbohD and AtRbohF have also been shown to function in salt tolerance by regulating the concentration of  $\text{Na}^+$  and  $\text{K}^+$  in the cell (Ma et al., 2012). AtRbohD is unique among other Rbohs in *Arabidopsis*, because it mediates rapid systemic signaling in response to various stimuli, and its expression is responsive to various stress conditions, such as drought, salt, heat and wounding (Miller et al., 2009; Suzuki et al., 2011). In addition, AtRbohD-derived ROS synthesis plays a role in salt acclimation (Xie et al., 2011).

The functions of Rbohs in other plant species have also been studied. NtNOX, a pollen specific Rboh in tobacco, positively regulates pollen tube tip growth (Potocký et al., 2007). PvRbohB in *Phaseolus vulgaris* plays positive role in rhizobial and mycorrhizal symbiosis, as well as lateral root development (Montiel et al., 2012; Arthikala et al., 2013; Montiel et al., 2013). In *Medicago truncatula*, the expression of *MtRbohA* is up-regulated in nodules; silencing *MtRbohA* leads to decrease in nitrogen fixation activity, suggesting that *MtRbohA* is a positive regulator in legume-rhizobia symbiosis (Marino et al., 2011).

Plant Rbohs are under the regulation of phosphorylation by protein kinases, as well as  $\text{Ca}^{2+}$ . Using a heterologous expression system with a mammalian cell line, human embryonic kidney (HEK) 293T, lacks  $\text{Ca}^{2+}$ -dependent ROS-producing activity, has provided detailed mechanisms of this regulation (Ogasawara et al., 2008). Chemically inducing  $\text{Ca}^{2+}$  influx into the

cell using ionomycin resulted in rapid ROS production in the AtRbohD-transfected cells, and this ROS production can be blocked by inhibiting Rboh enzyme activity or the absence of extracellular  $\text{Ca}^{2+}$  (Ogasawara et al., 2008). In fact,  $\text{Ca}^{2+}$  directly binds to the EF-hands in AtRbohD and induces conformational changes in this region, which is required for  $\text{Ca}^{2+}$ -induced ROS production (Ogasawara et al., 2008). In *Arabidopsis*, one major component of the core ABA signaling, the SnRK2.6/OST1, can physically interact and phosphorylate AtRbohF to induce its activity, which is required for ABA-induced stomatal closure (Sirichandra et al., 2009). AtRbohF can also be activated by  $\text{Ca}^{2+}$  in the HEK 293T cell line heterologous expression system, and this  $\text{Ca}^{2+}$  activation is dependent on the phosphorylation of AtRbohF (Kimura et al., 2012). The same regulatory mechanism has also been shown in rice (Takahashi et al., 2012). Also, AtRbohF can be phosphorylated by Calcineurin B-like (CBL)-interacting protein CIPK26 *in vitro*, and this interaction can induce AtRbohF-mediated ROS production (Drerup et al., 2013). In potato, two CDPKs can phosphorylate StRbohB at its N-terminus in a  $\text{Ca}^{2+}$ -dependent manner, and further induce ROS production (Kobayashi et al., 2007).

The MAPK signaling cascade is also involved in ROS signaling and regulate NADPH oxidases. As mentioned above, SnRK2.6/OST1 can phosphorylate AtRbohF for the production of  $\text{H}_2\text{O}_2$  in guard cells. In fact,  $\text{H}_2\text{O}_2$  can induce SnRK2.6/OST1 activity, and is required for the full activation of MAPKs MPK3 and MPK6, which can further induce stress-responsive gene expression (Kovtun et al., 2000; Rentel et al., 2004). In maize, ABA treatment leads to a biphasic induction of NADPH oxidase expression, where ZmMPK5 is required for the second phase induction, and is involved in a positive feedback loop in ABA-induced  $\text{H}_2\text{O}_2$  production (Lin et al., 2009). In tobacco, the ROS production by NADPH oxidase NbRbohB is also under the regulation of MAPK signaling during pathogen attack, where both NbRbohB expression and ROS production require MAPK signaling (Asai et al., 2008).

In mammalian cells, Rac/Rop GTPases are also important regulators that modulate NADPH oxidase activity by regulating electron transfer and the assembly of NADPH oxidase (Faris et al., 1998; Diebold and Bokoch, 2001; Bokoch and Zhao, 2006). In plants, rice OsRac1 has been shown to directly interact with the N-terminus of OsRbohB *in vivo*; this interaction promotes ROS production, and requires Ca<sup>2+</sup> binding to the EF-hands (Wong et al., 2007). In *Arabidopsis*, AtROP2 is a positive regulator of root hair tip growth, and the ROS production by AtRbohC/RHD2 during this process is dependent of AtROP2 (Jones et al., 2002; Jones et al., 2007). In pollen tubes, NADPH oxidase activity is regulated by Ca<sup>2+</sup> and signaling phospholipids such as PA, and also by Rac/Rop GTPases (Potocky et al., 2012).

The discovery of a cell wall Receptor-Like Kinase (RLK), FERONIA (FER), deepened the understanding of how ROP2-AtRbohC signaling pathway regulates root hair tip growth (Duan et al., 2010). Disruption in the FERONIA gene resulted in severe root hair growth resembling the *atrbohC/rhd2* mutant, and lower levels of ROS (Duan et al., 2010). First isolated as interacting protein of ROPGEFs, which are activators of Rac/Rop GTPases, FERONIA also physically interacts with ROP2, suggesting a possible complex formation by FERONIA, ROPGEF and ROP2 GTPase (Duan et al., 2010). FERONIA's closest homologs, pollen tube-specific RLKs, ANXUR1 (ANX1) and ANX2, act upstream of NADPH oxidases, AtRbohH and AtRbohJ, which are responsible for ROS production for pollen tube tip growth (Boisson-Dernier et al., 2013). Based on the fact that FERONIA also plays a role in plant-pathogen interactions as well as ANX functions in exocytosis, it is possible that these RLKs are cell surface sensors that trigger downstream signaling transduction through NADPH oxidases, then lead to cell wall changes, which further results in changes in growth (Cheung and Wu, 2011).

## ROS Regulate Growth via Class III Peroxidases

Root growth consists of two processes: cell division and cell elongation, where cell wall loosening or stiffening is required. The Class III peroxidases are heme-containing, plant-specific peroxidases, with abundant presence in the cell wall, and are important players in regulating the cell wall through ROS (Francoz et al., 2014) (Fig. 9). They control cell wall stiffening or loosening by regulating the levels of  $H_2O_2$  or producing  $OH^\cdot$ , respectively; and the balance of these different ROS molecules controlled by Class III peroxidases directly affect cell expansion (Schopfer, 1996; Schopfer, 2001; Liskay et al., 2004). Peroxidases can utilize  $H_2O_2$  as an oxidizer to promote cell wall cross-linking, lignin polymerization, which helps forming a rigid cell wall. On the other hand, peroxidases generate  $OH^\cdot$  from  $O_2^\cdot$  and  $H_2O_2$ , which can break the covalent bonds in the cell wall to loosen it (Schopfer, 2001; Francoz et al., 2014).

Class III peroxidases belong to a large gene family with diverse functions; there are 73 members in *Arabidopsis*, 138 in rice, 143 in *Brachypodium distachyon* (Hiraga et al., 2001; Passardi et al., 2005; Francoz et al., 2014). Most Class III peroxidases are secreted proteins and localize in the cell wall, and a small number of them can be found in the vacuole (Carter et al., 2004; Costa et al., 2008; Francoz et al., 2014). Some peroxidases are present in the lignified tissue and play a role in regulating lignification through  $H_2O_2$ . *Arabidopsis* extracellular peroxidase ATP A2 has been implied to function in lignification; mutants of this gene showed lower levels of lignin (Ostergaard et al., 2000). AtPrx37, AtPrx72 and AtPrx64 have also been shown to cause cell wall stiffening via lignification (Francoz et al., 2014). AtPrx37 is expressed in the vascular tissues; transgenic plants overexpressing this gene leads to increased phenolic cross-linking in the cell wall (Pedreira et al., 2011). AtPrx72 regulates lignin biosynthesis; disruption in AtPrx72 cause several developmental defects (Herrero et al., 2013). On the other hand, AtPrx33 and AtPrx34, two cell wall-localized Class III peroxidases, have positive effect in

root elongation, suggesting they may play a role in cell wall loosening through  $\text{OH}^-$  production (Passardi et al., 2006). AtPrx36, whose expression is under tight spatiotemporal control, only present during seed development at torpedo stage, has been shown to regulate the degradation of outer cell wall of seed coat for the secretion of mucilage, a pectic polysaccharide (Kunieda et al., 2013).

Recently, the mechanism of NADPH oxidase and cell wall peroxidase functioning together in the formation of Casparian strip in the endodermis has been revealed (Lee et al., 2013). The endodermal Casparian strip, which functions as a diffusion barrier, is made of lignin polymer (Naseer et al., 2012). AtRbohF, is surprisingly the only Rboh that functions in Casparian strip formation, by producing  $\text{H}_2\text{O}_2$  in the apoplast of the endodermis (Lee et al., 2013). Then the RbohF-produced  $\text{H}_2\text{O}_2$  will be utilized by an apoplast secreted peroxidase, Per64, which is recruited at the Casparian strip domain with RbohF by a Casparian strip domain protein CASP1, to promote lignin polymerization at this specific location (Lee et al., 2013).

A bHLH family transcription factor, UPBEAT1 (UPB1), regulates the expression of a subset of peroxidases in *Arabidopsis* root to modulate the balance of cell proliferation and differentiation (Tsukagoshi et al., 2010). UPB1 directly binds to the promoter of these peroxidases and down-regulates their expression, which then regulate ROS homeostasis between  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ , in root meristem and elongation zone respectively (Tsukagoshi et al., 2010). Changes of UPB1 expression lead to changes in the balance between these two ROS molecules, which can shift the boundary of cell proliferation and differentiation and result in changes of meristem size (Tsukagoshi et al., 2010).

### **ROS Function in ABA Signaling**

The function of ROS in ABA signaling has been studied extensively in guard cells in the process of ABA-induced stomatal closure. The mechanism of ROS mediated ABA signaling in guard cells has been discussed earlier in the section of ABA signaling. In this section here, the generation of ROS in guard cells, as well ROS-ABA signaling in roots will be introduced.

ROS are major secondary messengers in ABA-induced stomatal closure. Chloroplasts are thought to be the potential sources of ROS production in the guard cells, despite of the fact that guard cells contain fewer and smaller chloroplasts (Wang and Song, 2008). ROS can be produced during photosynthesis, and also from the conversion of excess light energy absorbed. In *Vicia faba*, H<sub>2</sub>O<sub>2</sub> levels are rapidly induced both by exogenous ABA or microinjected ABA in the guard cells, and the earliest induction can be observed in the region where chloroplasts localized (Zhang et al., 2001). Using DPI to chemically block the activity of NADPH oxidases block the increase of H<sub>2</sub>O<sub>2</sub> levels as well as ABA-induced stomatal closure; however, the H<sub>2</sub>O<sub>2</sub> produced by the chloroplasts are not affected (Kwak et al., 2003), which indicates that NADPH oxidases in guard cells are important contributors to ABA-induced H<sub>2</sub>O<sub>2</sub> production. Another direct evidence of ROS produced by NADPH oxidase is required for ABA-induced stomatal closure came from the studies done on AtRbohD and AtRbohF. As mentioned earlier, these two genes are expressed in guard cells and localize on plasma membrane; mutations in both two genes lead to complete insensitivity to ABA-induced ROS production as well as stomatal closure (Kwak et al., 2003). This indicates that plasma membrane localized NADPH oxidases contribute to ROS production by ABA in guard cells, and they are essential for stomatal closure triggered by ABA signaling.

Microarray study comparing transcriptome profiles between ABA and H<sub>2</sub>O<sub>2</sub> treated roots has shown that ABA and H<sub>2</sub>O<sub>2</sub> regulate the expression of genes involved in common biological processes, such as genes encoding membrane transporters and channels, and stress-responsive genes. About 30% of all H<sub>2</sub>O<sub>2</sub> -regulated genes are also ABA-regulated, but ABA and H<sub>2</sub>O<sub>2</sub>

signaling have their own unique downstream target genes (Wang et al., 2006), indicating ABA and H<sub>2</sub>O<sub>2</sub> signaling share common targets but also have their own distinct downstream players.

### **ROS and Legume-Rhizobia Symbiosis**

During legume-rhizobia symbiosis, which forms nodules for nitrogen fixation to occur, ROS have also been implicated to be the signaling molecules in this process. This symbiotic interaction involves the production of flavonoids by host legumes and signaling molecules by rhizobia, called Nodulation (Nod) factor (Fisher and Long, 1992). Upon the isolation of the *RIP1* gene in *Medicago truncatula*, which encodes a peroxidase, the role of ROS in nodulation has been investigated. Mt*RIP1* expression is induced by inoculation with rhizobia at 12 hour post inoculation, and also by H<sub>2</sub>O<sub>2</sub> very rapidly, in only 1 hour (Ramu et al., 2002). Interestingly, O<sub>2</sub><sup>-</sup> levels increase dramatically in 12 hour post inoculation, which was not observed in *dmi1* mutant, indicating ROS may function downstream of the symbiotic ion channel DMI1 that modulates Ca<sup>2+</sup> spiking (Ramu et al., 2002; Peiter et al., 2007). The exact function of RIP1 in early nodulation signaling is still not clear; however, its expression pattern co-localized nicely with O<sub>2</sub><sup>-</sup> localization indicates that it may be involved in regulate ROS that can modify cell wall structures, which allow root hair curling to happen.

Later, studies using H<sub>2</sub>O<sub>2</sub> efflux assay, performed on *M. truncatula* 1 cm root segments excised from the whole roots, showed a decrease in H<sub>2</sub>O<sub>2</sub> efflux within 30 minutes after NF application, and this effect can be blocked by the NADPH oxidase inhibitor DPI (Shaw and Long, 2003; Lohar et al., 2007). Also, the down-regulation of *Rboh* gene expression is also observed as early as 1 hour after NF addition (Lohar et al., 2007). On the contrary, a transient increase of intracellular ROS levels has been reported from the common bean *Phaseolus vulgaris* within



seconds of NF application (Cardenas et al., 2008). Elevated ROS levels are mainly detected in the tip of root hairs, peak at about 1 minute, and then decrease to basal levels-- this unique response of ROS is specific to NF, and sensitive to DPI treatment (Cardenas et al., 2008). These findings suggest that Rboh-produced ROS in the root hairs are highly responsive to NF, and they may play positive role in early nodulation signaling. Moreover, because Rboh have the common structural feature of EF-hands, which can interact with  $\text{Ca}^{2+}$  and trigger downstream signaling, it is possible that Rboh can interact with  $\text{Ca}^{2+}$  spiking during early nodulation signaling. ROS levels have also been observed to be increased at 4 hour post-inoculation, peak at 24 hour, and then start to decline (Peleg-Grossman et al., 2012).

In mature nodules of pea and alfalfa,  $\text{H}_2\text{O}_2$  was observed to be present in the infection threads, and more specifically, it is found to be surrounding bacteria within the infection threads, as well in the thread walls, and matrices of nodule invasion zone and nitrogen fixation zone (Rubio et al., 2004). It is also observed in the cell walls and intracellular space of the cortex adjacent to nodule meristem and invasion zone (Rubio et al., 2004). Interestingly, Cu/ZnSOD co-localize with  $\text{H}_2\text{O}_2$  (Rubio et al., 2004), suggesting at least some  $\text{H}_2\text{O}_2$  is produced by Cu/ZnSOD in nodules, but whether this  $\text{H}_2\text{O}_2$  might be used by peroxidases in order to modify cell wall is still not clear. It will be exciting to see if  $\text{H}_2\text{O}_2$  in nodules is regulating cell wall changes for infection thread progression.

In *M. truncatula* nodules,  $\text{H}_2\text{O}_2$  is detected in the inner cortex and nodule invasion zone (Peleg-Grossman et al., 2012). Microarray analysis on gene expression of *Sinorhizobium meliloti* inoculating DPI-treated roots revealed that some essential NF-signaling genes cannot be induced by inoculation (Peleg-Grossman et al., 2012), indicating ROS may be required for NF signaling gene expression.

### **1.3 ROOT AND NODULE DEVELOPMENT**

Plant root system consists of one primary root and lateral roots, branching out of the primary root. The primary root is established during embryogenesis, but lateral root development starts post-embryonically. Legumes have the ability to form another highly specialized root organ, nodules, where nitrogen-fixing bacteria enter and fix nitrogen into ammonia, which will be utilized by the plant host. In this section, I will review the current understanding of root and nodule development, and how plant hormones play a crucial role in regulating these processes.

#### **1.3.1 Root Development**

Plant roots are underground organs that not only provide structural support, but also are responsible for taking up water and nutrients for the whole plant. Roots are constantly exposed to various environmental signals and challenges, and they need to adjust their development or architecture for survival. Root system is very complex and diverse; this review section will be taking the advantage of the research done in the model species *Arabidopsis thaliana*, which has a relatively small and simple root structure.

#### **Patterning of the Root Apical Meristem**

The apical-basal polarity is established very early in embryogenesis, beginning with the first asymmetric cell division of the zygote, which produces a small apical cell and a bigger basal cell (Fig. 10). The apical cell will keep dividing and will produce the most part of the embryo; the basal cell divides horizontally to produce suspensor to connect the embryo to the maternal tissue,

and the top suspensor cell gives rise to hypophysis, which develops to root apical meristem (RAM) (Fig. 10). Asymmetric cell division occurs in hypophysis and generates a lens-shaped cell that becomes the quiescent center of the RAM, and a basal cell that becomes columella initials (Fig. 10). Auxin plays crucial role in embryonic root formation, supported by the facts that mutations in either auxin transporters or transcription factors functioning in auxin signaling lead to severe defects in embryonic root development. Auxin transport via the PIN proteins during embryogenesis is highly dynamic: after the zygote division, auxin is transported from basal cell into the apical cell via PIN7, then later the changes of PIN1 and PIN7 localizations lead to an auxin maximum in the top cell of the suspensor that becomes hypophysis and eventually the embryonic root (Friml et al., 2003). Auxin is essential for the specification of hypophysis and the root pole formation; mutants that lack the ability of auxin biosynthesis or polar transport usually exhibit no root phenotype (Friml et al., 2003; Cheng et al., 2007; Lau et al., 2012). Inhibiting auxin efflux using chemical inhibitors causes abnormal embryo structures, loss of the apical-basal polarity and the formation of embryonic root (Friml et al., 2003). The *WUSCHEL-RELATED HOMEODOMAIN* (*WOX*) genes that encode homeobox transcription factors are also determinant factors of embryonic root development. *WOX* genes express very early during embryogenesis; *WOX2* is even expressed in the egg cell before fertilization. *WOX* genes have dynamic and overlapping expression patterns, such as *WOX2* and *WOX8*, both of which are expressed in the zygote, but *WOX2* expression is restricted in the apical cell after the very first cell division, while *WOX8* transcript is only in the basal cell (Haecker et al., 2004). The dynamic expression pattern of *WOX* genes correlates nicely with the cell fate decision, indicating their role in the establishment of these decisions (Petricka et al., 2012). The *WOX* transcription factor family members exhibit functional redundancy in determining the apical-basal cell fate, and intersect with both auxin signaling and localization in the embryo (Breuninger et al., 2008). Although single mutants of the *WOX* genes did not show severe defects in embryonic development,

generating mutants of close homologs resulted in abnormal cell division and arrested development (Breuninger et al., 2008). Taken together, embryogenesis gives rise to embryonic RAM that provides new cells for post-embryonic root growth.

As mentioned above, RAM contains the QC, which are mitotically inactive cells, and initial cells surrounding the QC that can divide and produce different tissue types. Together, the QC and its surrounding initials form the stem cell niche (Petricka et al., 2012). The initial cells divide and form daughter cells, those of which on the shootward form lateral root cap, epidermis, cortex, endodermis and vasculature, while those on the rootward form columella root cap (Petricka et al., 2012). In *Arabidopsis* root, each cell type begins from an initial cell and exists as single cell file; cell position, rather than cell lineage, determines cell type (van den Berg et al., 1995). The QC in *Arabidopsis* RAM contains four cells, and functions in keep the surrounding columella initials dividing but not differentiating, apparently by producing a short-range signal to maintain the initial cells dividing (van den Berg et al., 1997). This signal could be *WOX5*, which is specifically expressed in the QC, represses columella differentiation (Sarkar et al., 2007; Petricka et al., 2012). QC specification can be divided into two pathways: the PLETHORA (PLT) pathway and the SHORTROOT (SHR)/SCARECROW (SCR) pathway (Fig. 11). The PLT genes, PLT1 and PLT2, which encode AP2-domain transcription factors, are expressed in the RAM stem cell niche and maintain the activity of the initial stem cells (Aida et al., 2004). Disruption of the PLT genes lead to altered cell division patterns in the RAM and then later on, severely decreased RAM and extremely short root (Aida et al., 2004). The gradient of the PLT proteins present in the RAM is shown to be important: high levels of PLT activity maintain the stem cell identity, and low levels of PLT promotes cell division or even differentiation at very low levels (Galinha et al., 2007). In the other pathway controlling QC specificity, SHR and SCR are GRAS family transcription factors, and SHR moves from the stele into the adjacent cell layers to turn on *SCR* expression, which is involved in positioning of the stem cell niche. Expression of *SCR* in the QC

is required for establishing the identity of QC and stem cell niche, and expressing *SCR* in other tissues cannot substitute its activity (Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003). Together, the SHR/SCR and the PLT pathways are major regulators in *Arabidopsis* root patterning and RAM specification.

### **Controls on Cell Division and Elongation in the Root**

New cells produced by the stem cell initials will pass three different developmental stages to reach maturity: in the meristematic zone (MZ), cells go through divisions; when they get to the elongation zone (EZ), they will rapidly elongate and then they will enter the differentiation zone (DZ), where they acquire specific functions (Petricka et al., 2012). The boundary of MZ and EZ is the transition zone (TZ); and the balance between cell proliferation and elongation determines the size of the meristem (Cederholm et al., 2012). Hormonal controls play major role in meristem size, where auxin and cytokinin and their interplays have shown to be major regulators of cell division and differentiation in the RAM. Auxin is synthesized mainly at the MZ, where it plays positive role in controlling cell division, and this auxin distribution is determined by efflux transporters, the PIN proteins. Mutations in the *PIN* genes, especially *PIN2*, lead to reduction of both MA size and the EZ size (Blilou et al., 2005). Cytokinin negatively regulates root meristem size in *Arabidopsis*, supported by the fact that cytokinin-biosynthetic or signaling mutants display larger meristems (Dello Ioio et al., 2007). Cytokinin does not affect QC and the stem cell niche specification, and it does not affect cell division rate; instead, cytokinin acts only at the TZ regulating cell differentiation, which in turn regulates the meristem size, determined by the balance of cell division and cell differentiation (Dello Ioio et al., 2007). Cytokinin signaling regulators, the ARR1 and ARR12 transcription factors, are specifically expressed at the TZ

(Dello Ioio et al., 2007); ARR1 directly binds to the promoter of SHY2/IAA3 *in vivo* and up-regulates its expression. SHY2/IAA3 is a member of the AUX/IAA family genes that act as auxin signaling inhibitors, auxin binding to the receptor triggers its degradation (Dello Ioio et al., 2008). Moreover, cytokinin-activated SHY2/IAA3 via ARR1 not only restricts the expression of three *PIN* genes, *PIN1*, *PIN3* and *PIN7* to the vasculature of the TZ, but also functions in auxin-induced cytokinin biosynthetic gene expression (Dello Ioio et al., 2008). These findings indicate auxin and cytokinin act antagonistically via ARR1 and SHY2/IAA3 at the TZ to balance cell division and differentiation, thus regulate the meristem size.

In the EZ, cells rapidly elongate unidirectionally, which contributes to primary root growth. Cells need to go through many changes to prepare elongation, such as central vacuole development, cytoskeleton rearrangement, and cell wall changes (Petricka et al., 2012). Cell wall expansion is very complex; it could be regulated at many different levels, such as cell wall biosynthesis and assembly, as well as the activity of wall loosening enzymes. Expansins can directly induce cell wall extension by disrupting hydrogen bonds between cellulose microfibrils, and their positive role in cell elongation has been revealed in many species such as *Arabidopsis*, soybean and rice (McQueen-Mason and Cosgrove, 1994; Lee et al., 2003; Lin et al., 2011; ZhiMing et al., 2011). Also, xyloglucan endotransglycosylase (XET) act at the EZ to regulate root elongation (Vissenberg et al., 2000). XET can cleave xyloglucans, which are the major hemicellulose connecting cellulose microfibrils in the primary cell wall, and rejoin the newly-formed ends. Studies have shown that some XETs play positive role in cell elongation, although another study observed that adding purified XET proteins result in a decrease of cell elongation (Osato et al., 2006; Maris et al., 2009). Microtubules are another factor that control cell elongation. The organization of MT in the cells of EZ is transverse to the direction of the elongation (Ehrhardt and Shaw, 2006); chemically disrupting MT results in radial swelling (Sugimoto et al., 2003).

## Lateral Root Development

Unlike primary roots, which are established during embryogenesis, lateral roots (LRs) development is a post-embryonic process and determines the root architecture (Tian et al., 2014). In *Arabidopsis*, LRs are initiated specifically from the pericycle cells adjacent to the xylem poles, and the LR development can be divided into eight developmental stages, starting from asymmetrical cell divisions in a subset of pericycle cells called the founder cells, which will further undergo anticlinal division to form LR primordia (LRP) (Malamy and Benfey, 1997). Then more cell divisions occur in the LRPs; at later stages the LRPs resemble primary root tips. The final emergence of LRs is mainly from cell expansion, and then the LR meristem becomes activated and LRs begin to grow (Malamy and Benfey, 1997; Petricka et al., 2012). Different LRs emerge along the primary root, the spacing between LRs is variable, with the younger LRs rootward from the older LRs (Dubrovsky et al., 2006; Petricka et al., 2012).

LR formation is under the control of hormonal signaling. Auxin is by far the best studied major hormone in regulating LR formation. The oscillation of auxin responses primes pericycle cells to form pre-branch sites (Moreno-Risueno et al., 2010). In *Arabidopsis*, using the synthetic auxin response promoter, DR5, the auxin response can be detected even before the very first formation of founder cells. Auxin response indicated by DR5:GUS reporter showed that auxin response is dynamic: it is present in all cells of LRP in the early stages, and becomes a gradient at later stages with the highest in the tip of the LRP (Benková et al., 2003). Auxin promotes pericycle cell cycle activation through D-type cyclin (Himanen et al., 2002). Mutation in *IAA14*, a *AUX/IAA* family gene, blocks cell division at the G1 to S phase transition, thus blocks LR initiation, so that *irr14* mutants do not have LRs (Fukaki et al., 2002). However, only triggering

cell cycle activation is not enough to produce normal LR in *irr14* mutant, indicating IAA14-mediated auxin signaling functions not only in cell cycle activation, but also in the cell fate specification (Vanneste et al., 2005). Moreover, IAA14 interacts with ARF7 and ARF19 (Fukaki et al., 2005), together they can regulate different downstream targets, to control many other aspects of LR development, including up-regulating cell wall remodeling genes, as well as auxin homeostasis or localization (Lavenus et al., 2013). Auxin signaling is such a complex network with numerous players, each of those can regulate their own set of targets; as a result, how all these regulations coordinate together is still under investigation.

Cytokinin has been known to inhibit lateral root development; many *Arabidopsis* cytokinin receptor or signaling mutants have more root branching compared to wild-type (Werner et al., 2001; To et al., 2004; Riefler et al., 2006). Recent studies have closely examined the effect of cytokinin on different stages of LR development. Cytokinin has negative impact on the very early developmental stages, including LR initiation and priming (Bielach et al., 2012). Exogenous cytokinin leads to reduced number of LRPs, indicating that cytokinin is affecting LR initiation (Laplaze et al., 2007). LRP formation is a highly organized process; cell division at a certain stage is highly oriented. Cytokinin has been shown to perturb the organization of cell divisions leading to the LRP formation (Laplaze et al., 2007). In fact, cytokinin specifically acts in the pericycle founder cells of xylem pole, but not in LRPs (Laplaze et al., 2007). Cytokinin does not have an effect on initiated LRP, but positively regulates LR elongation (Li et al., 2006). Cytokinin application alters auxin response and the establishment of auxin maximum indicated by DR5:GUS reporter during LR initiation (Laplaze et al., 2007). Also, cytokinin represses both the spatial pattern and levels of *PIN* gene expression (Laplaze et al., 2007). A recent study also showed that cytokinin regulates the polarity of PIN1-mediated auxin efflux transport in LR formation (Marhavý et al., 2014).



## ABA Regulates Root Development

ABA also plays very important role in regulating root development. It has been long known that ABA inhibits root elongation, in both primary and lateral roots (Pilet, 1975; De Smet et al., 2003). However, ABA's positive role in regulating growth has also been implied from the fact that some ABA deficient mutants have growth defects. For example, *aba2* mutants, which is defective in ABA biosynthesis, exhibit severe growth retardation (Cheng et al., 2002). In *Arabidopsis*, it has been shown that ABA may promote growth by maintaining RAM function. ABA promotes stem cell maintenance in the RAM of primary root, by promoting QC staying quiescent as well as suppressing the stem cells from differentiating (Zhang et al., 2010). *Arabidopsis* seedlings grown ABA have larger DZ and EZ, but interestingly, ABA increases cell number in the DZ but reduced the number of cells that are actually dividing (Zhang et al., 2010). ABA induces the expression of *WOX5*, *PLT2* and *MP*, all of which play significant role in the RAM (Zhang et al., 2010).

The inhibition of ABA on lateral root development is reversible, and is specific to a certain stage: only after the emergence of the LRP but before the activation of the meristem (De Smet et al., 2003). ABA suppresses certain cell cycle gene expression and auxin responses; adding auxin cannot rescue arrested LRs caused by ABA, indicating ABA may act in auxin-independent pathway (De Smet et al., 2003). *ABI1*, *ABI2* and *ABI3* appear to only play a minor role in ABA-inhibition of LR growth, suggesting the existence of novel signaling components that function in this process (De Smet et al., 2003).

However, it is obvious that ABA intersect with other plant hormones in regulating root development. Some evidence of the interplay between ABA, cytokinin and auxin has been revealed. *ABI4*, the ABA-inducible AP2-family transcription factor, negatively regulates LRP formation and LR elongation, by controlling auxin and cytokinin distributions (Shkolnik-Inbar

and Bar-Zvi, 2010). ABI3, a transcription factor containing B3 binding domain and functioning in ABA signaling, is weakly expressed in the dividing pericycle cells during the LR initiation stages, but absent after the emergence of LRP (Brady et al., 2003). *abi3* mutants exhibit decreased sensitivity to auxin-induced LR formation, and *ABI3* expression in roots can be up-regulated by synthetic auxin 2, 4-D (Brady et al., 2003). On the other hand, players in auxin signaling pathway have also been shown to be under ABA's regulation, suggesting another level of crosstalk between ABA signaling and auxin signaling. Auxin Responsive Factor 2 (ARF2), positive regulator in auxin signaling, is also regulated by ABA at expression levels, and it is important for ABA-inhibited root growth (Wang et al., 2011). Under *arf2* mutant background, the accumulations of auxin efflux facilitators, the PIN proteins, can be inhibited by ABA, which may explain the increased sensitivity of *arf2* mutants in ABA-inhibited root growth (Wang et al., 2011). ABA receptor PYL8, interacts with transcription factor MYB77 and enhance its binding to the promoters of auxin-responsive genes, in the process of LR growth recovered from ABA's inhibition (Zhao et al., 2014).

Root development is extremely sensitive to the environment; since ABA plays major role in mediating stress responses, therefore ABA-regulation in root development connecting various environmental conditions has been studied. It has been shown that ABA maintains root growth in maize seedlings under low water potentials, by restricting ethylene production (Saab et al., 1990; Spollen et al., 2000). Under salt stress, ABA signaling in the endodermal cell layer is necessary for post-emergence LR growth inhibition, and GA acts parallel to ABA antagonistically in the endodermis (Duan et al., 2013). Nitrate is another important factor that regulates LR development, and it has been shown that ABA is crucial for the nitrate inhibition on LR development. High concentrations of nitrate (10 or 50 mM) inhibit both LR number and LR elongation, but this inhibition is strongly attenuated in ABA-signaling mutant *abi4* and ABA-biosynthesis mutants *aba2* and *aba3* (Signora et al., 2001). However, it is notable that most *abi*

mutants did not have altered LR response to high nitrate (Signora et al., 2001), indicating that ABA-mediated nitrate inhibition on LR development may signal through novel proteins. Localized 1 mM nitrate has stimulatory effect on LR development (Zhang and Forde, 1998, 2000); this stimulation is reduced in *aba1-1* mutant but enhanced in most *abi* mutants (Signora et al., 2001). Overall, ABA's effect on nitrate-mediated LR development is intriguing, but clearly the mechanisms of how ABA regulates the dual functions of nitrate in LR development are different between low concentrations of localized nitrate and evenly distributed high concentrations of nitrate.

### **1.3.2 Symbiotic Nodule Development**

The symbiotic interaction between legumes and rhizobia leads to the formation of a specialized type of organ in legume roots: the nodule, in which nitrogen-fixing bacteria will convert triple-bonded dinitrogen into ammonia, a form of nitrogen that can be transported and utilized by plants (Oldroyd and Downie, 2008). Nodule organogenesis is a highly coordinated process that requires bacterial infection in the root epidermis, and cell divisions several cell layers below the infection site in the inner cortex (Oldroyd et al., 2011). This review will introduce nodule signal transduction, bacterial infection, nodule organogenesis and its hormonal control, focusing primarily on the model legume, *Medicago truncatula*.

#### **Model Legume *Medicago truncatula***

The legume family is large and diverse, including many important crop species, such as soybean, alfalfa, and pea. However, the large genome sizes of these crop legume species have made genetic characterization very difficult. *Medicago truncatula*, a close relative of alfalfa

(*Medicago sativa*), on the other hand, has a relatively small, diploid genome. It has a short life cycle, self-fertile, and also exhibits high levels of natural diversity (Barker et al., 1990; Frugoli and Harris, 2001). This has made *M. truncatula* a great model legume species for research.

Many tools and resources have been developed for *M. truncatula* and are publicly available. The *Medicago truncatula* Gene Expression Atlas (MtGEA) was established by combining microarray data collected from a wide range of different tissue types and treatments (Benedito et al., 2008). Recently, the genome sequence data of *M. truncatula* has been released (Young et al., 2011; Tang et al., 2014). Also, proteomics and phosphoprotein databases are also available (Colditz and Braun, 2010). A large-scale *Tnt1* retrotransposon mutant population and several EMS TILLING populations have also been generated and made mutant isolation for gene of interest possible (Tadege et al., 2008; Rogers et al., 2009).

### **Nod Factor Signaling Transduction**

Nodulation in legumes is activated upon the perception of Nodulation (Nod) factors (NF) produced by the bacteria. Nod factors consist of chitooligosaccharide backbone, made of four or five N-acetylglucosamine (GlcNAc) residues with  $\beta$ 1-4 linkages (Dénarié et al., 1996). This lipochitooligosaccharide (LCO) can be further decorated on the GlcNAc subunits; the length and the degree of saturation of the N-acetyl group can also be modified, resulting in species-specific Nod factor, which functions in determining the specificity of this symbiotic relationship with their host legumes (Oldroyd, 2013). Nod factor is recognized by the Nod factor receptor, LysM receptor-like kinases. Two forms of LysM receptor-like kinases have been found: the LysM I clade including the *Lotus japonicus* Nod Factor Receptor 1 (NFR1) and *Medicago truncatula* LysM receptor kinase 3 (LYK3), and the LysM II clade including *L. japonicus* NFR5 and *M.*

*truncatula* Nod Factor Perception (NFP) (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006). It has been recently shown that in *L. japonicus*, NFR1 and NFR5 can interact with each other and directly bind to Nod factor at nanomolar range (Madsen et al., 2011; Broghammer et al., 2012). Both NFR1 and NFR5 are required for Nod factor signaling and act upstream of the common symbiotic pathway of nodulation and mycorrhization (Radutoiu et al., 2003). In vitro assay did not detect any kinase activity of NFR5 kinase domain, but that of NFR1 is essential in Nod factor signaling in vivo and kinase activity in vitro (Madsen et al., 2011). This suggests that these receptor kinases may function as heterodimers or large-order heterocomplexes (Oldroyd, 2013) (Fig. 13).

Downstream of Nod factor receptors in the Nod factor signaling pathway is the Symbiosis Receptor-like Kinase/Doesn't Make Infections 2 (SYMRK/DMI2). DMI2 encodes a receptor-like kinase that contains an extracellular domain with leucine-rich repeats (Endre et al., 2002), and it is required for earliest root hair physiological responses and  $\text{Ca}^{2+}$  oscillations (Catoira et al., 2000; Wais et al., 2000). The exact role of SYMRK.DMI2 is still not clear. It is predicted that in *M. truncatula*, upon the binding of Nod factor, LYK3, NFP and Symbiosis Receptor-like Kinase/Doesn't Make Infections 2 (SYMRK/DMI2) forms a receptor complex, which activates  $\text{Ca}^{2+}$  oscillations in the nucleus (Oldroyd, 2013) (Fig.13).

Symbiotic  $\text{Ca}^{2+}$  oscillations, named  $\text{Ca}^{2+}$  spiking, occur in the root hair cells in response to Nod factor (Ehrhardt et al., 1996), requires cation channel DMI1 (as Doesn't Make Infections 1), which is localized on the inner nuclear membrane (Riely et al., 2007). DMI1 is preferentially permeable to potassium, so that it probably does not function as  $\text{Ca}^{2+}$  channel; the current hypothesis is that DMI1-mediated potassium flux regulates  $\text{Ca}^{2+}$  channel opening and balances the charge of the nuclear membrane after the flow of  $\text{Ca}^{2+}$  from the ER store into the nucleoplasm (Peiter et al., 2007; Oldroyd, 2013). Mathematical modeling predicts that a calcium-ATPase, MCA8, functioning as calcium pump, together with DMI1 and an unknown calcium channel are

sufficient to produce  $\text{Ca}^{2+}$  oscillations (Capoen et al., 2011; Oldroyd, 2013). These  $\text{Ca}^{2+}$  oscillations are perceived by  $\text{Ca}^{2+}$ /Calmodulin-dependent protein kinase (CCaMK), or DMI3, which can be activated by  $\text{Ca}^{2+}$  binding to its EF-hands as well as calcium and calmodulin (CaM) complex binding to the CaM-binding domain (Levy et al., 2004; Gleason et al., 2006; Tirichine et al., 2006). CCaMK interacts with and phosphorylates CYCLOPS, a nuclear-localized Interacting Protein of DMI3 (IPD3) in *M. truncatula* (Messinese et al., 2007; Horvath et al., 2011) (Fig. 13).

Downstream of CCaMK and IPD3 are the transcription factors Nodulation Signaling Pathway 1 and 2 (NSP1 and NSP2), Ethylene Response Factor Required for Nodulation 1 (ERN1), Nodule Inception (NIN), and Nuclear Factor Y subunit A (NF-YA), all of which regulate Nod factor-responsive gene expression (Oldroyd and Long, 2003; Kaló et al., 2005; Murakami et al., 2006; Cerri et al., 2012; Laloum et al., 2014) (Fig. 13). The two GRAS family transcription factors, NSP1 and NSP2, form heterocomplexes, although only NSP1 has DNA-binding domains for binding to the promoters of Nod-factor-inducible genes (Hirsch et al., 2009). The induction of the transcription of NIN and ERN1 in response to Nod factor, another two important transcription factors in Nod factor signaling, requires NSP1 and NSP2 (Marsh et al., 2007; Hirsch et al., 2009). Both NSP1 and ERN1 can induce the expression of *EARLY NODULIN 11* (*ENOD11*), an early nodulin gene expressed initially in the epidermis, by directly binding to its promoter, but at different locations (Journet et al., 2001; Cerri et al., 2012). ERN1 binding to the “Nod factor box” of ENOD11 promoter is required for early Nod factor signaling (or “preinfection”), while the regulation by the NSP1 and NSP2 complex of binding to the “Infection-related” promoter sequences of ENOD11 mediates subsequent rhizobial infection (Cerri et al., 2012). Recently, two CCAAT-box binding transcription factors, NF-YA1 and NF-YA2 have been shown to be essential for early nodulation signaling by directly activating ERN1, and also function in later stages of rhizobial infection and nodule meristem formation (Combiér et al., 2006; Combiér et al., 2008; Laporte et al., 2013; Laloum et al., 2014). NF-YA1 is expressed

as early as 6 hour- post inoculation in the epidermis and controls bacterial infection (Laporte et al., 2013; Laloum et al., 2014). In *L. japonicus*, NF-YA1 and NF-YB1 regulate cell division during nodule organogenesis and they are direct transcriptional targets of NIN (Soyano et al., 2013). In common bean *Phaseolus vulgaris*, a C subunit of the Nuclear Factor Y, NF-YC, also plays key role in nodule organogenesis and bacterial infection, and it interacts with a new GRAS family TF, SIN1, which also regulates in lateral root elongation, nodule organogenesis as well as infection progression (Zanetti et al., 2010; Battaglia et al., 2014). Together, different TFs downstream of  $\text{Ca}^{2+}$  spiking perception function both cooperatively and distinctively to regulate downstream gene expression in early Nod factor signaling pathway.

### **Bacterial Infection**

Rhizobia have the ability to attach to legume root hairs, which is the first step of the association with their legume host (Oldroyd and Downie, 2008). Plant lectins are located on the growing tip of the root hairs enhance this attachment (Smit et al., 1992). Rhizobial infection of *M. truncatula* roots occurs via an infection thread (IT), which starts in the growing root hairs. Upon the perception of Nod factor, legume root hairs will undergo an interruption of tip growth, followed by root hair swelling, branching and curling back at 180° to 360° on itself (Ehrhardt et al., 1996; Esseling et al., 2003). This root hair response leads to the attached NF-producing bacteria becoming entrapped in the root hair curl, forming an infection pocket. The root hair deformation is associated with changes in the actin and microtubule cytoskeleton, as well as cell wall remodeling (Brewin, 2004; Oldroyd and Downie, 2008). Root hair curling requires the Nod factor receptor, but not Nod factor signaling with  $\text{Ca}^{2+}$  spiking, which means that Nod factor

perception leads to two downstream events: root hair deformation and  $\text{Ca}^{2+}$  spiking that leads to gene expression changes (Miwa et al., 2006; Oldroyd et al., 2011) (Fig. 12).

The next step in infection is the inward-directed growth of plant cell wall material forming a tubular structure leading from the infection pocket. The entrapped bacteria divide in the infection pocket, forming colonies called infection foci, and subsequent division populates the IT as it grows (Oldroyd et al., 2011). Plant cell wall degradation at the growing tip is necessary to prevent cell rupture, and together with the extended plasma membrane, the IT grows as a tube, but still topologically outside of the plant cell, separated from the cytoplasm by both a plasma membrane and the cell wall. The growing IT then reaches the base of the root hair cell, and, as it approaches the nucleus, the adjacent cortical cell repositions itself and forms a cytoplasmic bridge called a pre-infection thread (pre-IT). This aligns with the growing IT tip, accompanied by the localized plant cell wall degradation, allowing the IT to continue growing toward the next cell layer. This process repeats, allowing the IT to keep growing until it reaches the dividing cortical cells in the inner cortex that forms nodule primordium (Fig. 12). The IT penetrates host cells and eventually releases rhizobia, which then are internalized and surrounded by host membrane, forming vesicles called symbiosomes. Within the symbiosome, the infecting bacteria then differentiate into a bacteroid, under the control of plant-derived, nodule-specific, cysteine-rich (NCR) peptides (Van de Velde et al., 2010). Defective in Nitrogen Fixation (DNF1) encodes a signal peptidase, is required to target these NCR peptides to symbiosomes, where they are incorporated into the bacteroids and drive the bacteroid development (Wang et al., 2010).

### **Nodule Organogenesis**



Legume-rhizobial symbiosis leads to the formation of nodules, where nitrogen fixation takes place. There are two types of nodule development that occur in different legume species. *Medicago truncatula* develop indeterminate nodules, which have a tip-growing nodule meristem, while *Lotus japonicus* develop determinate nodules that lose the nodule meristem at an early stage. In this review section, I will introduce indeterminate nodule development in model legume *M. truncatula*, in which nodules originate from inner cortical cells.

*M. truncatula* forms indeterminate nodules that exhibit a developmental gradient inside the nodules, with youngest tissue at the tip, where the nodule meristem produces new cells, and the oldest tissue at the base of the nodules next to the root. Nodule organogenesis starts from the activation of the mitotic cell cycle, which has been recently shown to occur in the inner and middle cortical cells, and pericycle cells. The third layer of cortical cells gives rise to the nodule meristem, while the 4<sup>th</sup> and 5<sup>th</sup> layer give rise to the infected cells (Xiao et al., 2014) (Fig. 14). Cortical cell divisions lead to the formation of a nodule primordium, which the ITs eventually reach and release bacteria into. While the root epidermis regulates bacterial infection, cortical cells control nodule formation. Making a functional nodule is a highly coordinated process of both developmental processes, which are considered to be different responses to Nod factor, and are mediated by different regulators. Moreover, bacterial infection can occur without nodule organogenesis; nodule formation can be induced in the absence of bacterial infection. Autoactivation of CCaMK by removing its autoinhibition domain leads to the formation of spontaneous nodules without bacterial infection (Gleason et al., 2006).

The plant hormone cytokinin plays a crucial role in nodule organogenesis. A Nod<sup>-</sup> rhizobium strain carrying a plasmid which constitutively expresses the bacterial *trans*-zeatin secretion gene, which catalyzes the last step of cytokinin synthesis (Cooper and Long, 1994), can induce nodule development on alfalfa roots, indicating that cytokinin can induce nodule organogenesis in the absence of rhizobial infection (Cooper and Long, 1994). Application of

cytokinin also resulted in the formation of nodule-like structures (Bauer et al., 1996; Heckmann et al., 2011). Many key components in cytokinin signaling during nodulation have been identified. In *L. japonicus*, cytokinin signaling has been shown to be required for initiating cell division in the cortex by regulating NIN (Murray et al., 2007). The cytokinin receptor mutant *hit-1* is defective in cortical cell division and nodule formation, but has more infection events (Murray et al., 2007). Knocking down the expression of *MtCRE1*, which encodes the cytokinin receptor, leads to a strong reduction in both cortical cell division and nodule number (Gonzalez-Rizzo et al., 2006).

Cytokinin application can strongly induce the expression of Nod factor signaling genes in the cortex, including *NSP1*, *NSP2*, *NIN*, and *ERN1*; *MtCRE1* is required for this transcriptional induction (Plet et al., 2011). *MtRR4*, a Type-A Response Regulator (RR), a key component of the cytokinin signal transduction pathway, is strongly up-regulated by cytokinin, and is also inducible by rhizobia inoculation (Gonzalez-Rizzo et al., 2006). Using the *MtRR4* promoter: GUS fusion, the cytokinin response during nodule organogenesis has been revealed: it is first present in the inner cortex and pericycle at very early stage, then in the dividing cortical cells and nodule primordium (Plet et al., 2011). In mature nodules, cytokinin response indicated by *RR4* expression is restricted to the nodule meristem and infection zone, indicating the role of cytokinin in controlling cell division and differentiation (Plet et al., 2011). Ectopic expression of *MtRR9*, another Type-A RR, leads to arrested nodule primordia and more lateral roots. Since Type-A RRs are considered to be negative regulators of cytokinin signaling, this finding suggests *RR9*-mediated cytokinin signaling plays a positive role in nodule formation (Op den Camp et al., 2011). The EFD transcription factors, which contain ERF1, 2 and 3, are required for nodule formation partially by activating *MtRR4* (Middleton et al., 2007). The Type-B RR, *MtRR1*, directly targets *MtRR4* and *NSP2*, an essential transcription factor in nodulation signaling (Ariel et al., 2012). *MtRR1* binds to the promoters of these two genes and turn on their expression (Ariel

et al., 2012). These results suggest that cytokinin regulates nodule organogenesis by signaling directly through nodulation signaling genes. The fact that MtRR1 is predicted to also bind to the promoter of Cytokinin Oxidase 1 (CKX1), encoding an enzyme that degrades cytokinin and negatively regulating nodule development (Ariel et al., 2012), suggests the presence of negative feedback regulation by cytokinin signaling during nodulation (Fig. 15).

Polar auxin transport has been reported to play a role in nodule organogenesis. In several studies, applying auxin polar transport inhibitors induces the formation of nodule-like structures, in the absence of rhizobia, as well as inducing the expression of some early nodulin genes (Hirsch et al., 1989; Rightmyer and Long, 2011). Using the DR5:GUS auxin response reporter from *Arabidopsis* exhibited GUS activity in developing nodules, and knocking down the expression of MtPIN genes led to reduced nodule numbers (Huo et al., 2006). The expression of genes encoding auxin efflux transporters, the PIN proteins, is induced by Nod factor. Cytokinin appears to play a role in regulating auxin transport during nodule formation. The expression of PIN genes in *M. truncatula* can be induced by cytokinin treatment, although it affects different PINs differently (Plet et al., 2011). The cytokinin receptor mutant *cre1* has been shown to have stronger PIN protein signals in the cortex (Plet et al., 2011). These results suggest a possible function of cytokinin in regulating auxin polar transport during nodule formation.

ABA has been shown to negatively regulate nodulation. Exogenous ABA inhibits *S. meliloti*- or Nod factor-induced gene expression and Ca<sup>2+</sup> spiking; inhibiting ABA signaling by expressing the *Atabi1-1* dominant mutation leads to hypernodulation and insensitivity to ABA (Ding et al., 2008). ABA suppresses cytokinin's induction of nodulation signaling genes *NIN* and *ENOD40* (Ding et al., 2008), indicating that the role of ABA in nodulation is antagonistic to that of cytokinin. Interestingly, ABA and cytokinin also regulate LR formation in opposite ways, with ABA inducing LR development in legumes and cytokinin inhibiting it. It is possible that the ratio of cytokinin to ABA is important for LR and nodule development (Ding et al., 2008).

### 1.3.3 The *Medicago truncatula* *LATD/NIP/NPF1.7* Gene Regulates Root and Nodule Development

The *M. truncatula* *lateral root organ-defective* (*latd*) mutant was first isolated in a screen for nodulation defective mutants (Bright et al., 2005). *latd* mutants have an extremely short primary root, with no elongated LR. Root hairs are also short in *latd* mutants, as compared to wild type (Bright et al., 2005). The root meristems in both primary and lateral roots are disorganized, and this phenomenon is developmental age related, since the *latd* root can grow similarly to wild type up to about 5 days, then the growth rate declines and the root eventually arrests (Bright et al., 2005; Liang et al., 2007). Nodules on *latd* mutants are small, white and round-shaped, and exhibit no nitrogen fixation activity (Bright et al., 2005). Rhizobial infection in *latd* mutants can occur, but the process is incomplete. The most advanced IT can get to the inner cortex or the nodule primordia, but the extent of IT ramification is severely reduced. At a stage where the wild-type developing nodule is filled with rhizobia, *latd* nodules are relatively empty, with bacteria apparently restricted to infection threads (Bright et al., 2005). Another allele of *latd*, *nip-1*, was isolated in a separate screen for nodulation mutants (Veereshlingam et al., 2004). The *nip-1* mutants also have a short primary root and fewer LRs, as well as small, white nodules (Veereshlingam et al., 2004). The *nip-1* mutant does exhibit a range of root phenotypes, with some homozygotes having longer primary roots and elongated lateral roots, which was not observed in *latd* homozygotes. *nip-1* mutant nodules are also small and white, with very few bacteria inside, and does not fix nitrogen (Veereshlingam et al., 2004). Rhizobial induction of *ENOD2* and *ENOD8*, which are expressed in nodule parenchyma and infected nodule cells, respectively, is abolished in *nip-1* mutants (Veereshlingam et al., 2004), indicating a defect in

nodule development. Both *latd* and *nip-1* mutants also exhibit hyperaccumulation of polyphenolic compounds (Veereshlingam et al., 2004; Bright et al., 2005). The third allele, *nip-3*, which is also defective in nodulation, has the least severe phenotype among the three alleles. The nodules in *nip-3* mutants are significantly more developed and have detectable nitrogen-fixation activity, although it is still significantly lower than that of wild-type, and release of bacteria from infection threads is still strongly reduced (Teillet et al., 2008). In addition, primary root length is decreased, as it is in *latd* and *nip-1* mutants, but lateral root development appears wild-type (Yendrek et al., 2010). In summary, the characterization of mutant phenotypes in *latd* and its alleles *nip-1*, *nip-3* indicates that LATD/NIP is required for normal root and nodule development.

The MtLATD/NIP/NPF1.7 gene has been cloned and shown to encode a nitrate transporter (Yendrek et al., 2010). The *latd* mutation caused a premature stop codon in the fourth exon, while the *nip-1* and *nip-3* mutations each caused amino acid substitution. The LATD/NIP gene is expressed in various tissue types, but expression is especially high in the root tip and nodule meristem. The MtLATD/NIP/NPF1.7 gene belongs to the large NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER (NRT1/PTR) family, which has been recently renamed NPF as a unified nomenclature (Léran et al., 2014). The plant NPF family proteins are predicted to have twelve transmembrane domains, and display sequence homology with proteins present in many other kingdoms such as bacteria, fungi and animals. (Léran et al., 2014). Members of the NPF family have been shown to transport a range of various molecules, such as dipeptides, dicarboxylates glucosinolates, and some members can transporter both nitrate and plant hormones as auxin and ABA (Léran et al., 2014). The first NPF family member characterized was *Arabidopsis thaliana* NRT1.1/CHL1/NPF6.3 which transports nitrate. Later, it was shown to also facilitate auxin uptake (Tsay et al., 1993; Krouk et al., 2010). The mechanism of NRT1.1 functioning in root growth is proposed as: NRT1.1 functions as a nitrate sensor that inhibits LR growth under low nitrate conditions by transporting auxin out of the cell (Krouk et al.,

2010). AtNRT1.2/AT1/NPF4.6, whose expression pattern is correlated with the sites of ABA biosynthesis, transports nitrate and mediates ABA uptake, and nitrate does not compete with ABA as a substrate (Kanno et al., 2012; Kanno et al., 2013). A possible mechanism of AtNRT1.2 is that it can mediate ABA uptake at the sites of ABA biosynthesis (Kanno et al., 2012), which could lead to rapid response to ABA-mediated stress responses. Functional analysis carried out in *Xenopus laevis* oocytes has revealed that LATD/NIP is a high affinity nitrate transporter (Bagchi et al., 2012). Whether LATD/NIP can also transport other molecules as some of the NPF members remains unknown.

Interestingly, exogenous ABA can rescue root growth defects in *latd* mutants (Liang et al., 2007). ABA application not only restores the growth of primary root elongation, but also rescues LR elongation defects. *latd* mutants grown on ABA therefore have a longer primary root, normal elongated LRs, as well as normal root hairs. ABA increases the number of elongated LRs in *latd* mutants, while increases the number of both emerged and elongated LRs in wild-type (Liang et al., 2007). In the RAM, ABA treatment significantly restores the morphology and the RAM organization of *latd* mutants (Liang et al., 2007). However, the primary and lateral root growth cannot be rescued by global nitrate application (Yendrek et al., 2010), nor can expression of the NRT1.1/NPF6.3 nitrate transporter fully rescue root and nodule meristem function, suggesting an additional function for MtLATD/NIP in addition to nitrate transport (Bagchi et al., 2012). *latd* mutants also exhibit ABA-insensitive phenotypes in seed germination and stomatal closure (Liang et al., 2007), indicating that LATD/NIP is also required for ABA signaling. The mechanism of how ABA rescues *latd* growth defects is not clear, although it is interesting to speculate that MtLATD/NIP may be able to also transport ABA. ABA does not rescue the nodulation phenotype of *latd* mutants, most likely because ABA inhibits nodulation (Liang et al., 2007; Ding et al., 2008).

## SUMMARY

This chapter provides a review of the physiological functions and molecular mechanisms of the plant hormone ABA in the aspect of stress responses, as well as the regulation of root and nodule development. Model legume *M. truncatula* LATD/NIP is a recently discovered gene that plays an important role in root and nodule formation, as well as in mediating nitrate transport and ABA signaling. The functions of ABA in legumes have not been examined as extensively as in *Arabidopsis*. How ABA may coordinate nitrate signaling, in root and nodule formation in legumes, especially whether and how ABA may mediate legume root and nodule development in response to environmental stresses also needs to be investigated. The following chapters in this dissertation will present my research addressing these questions.

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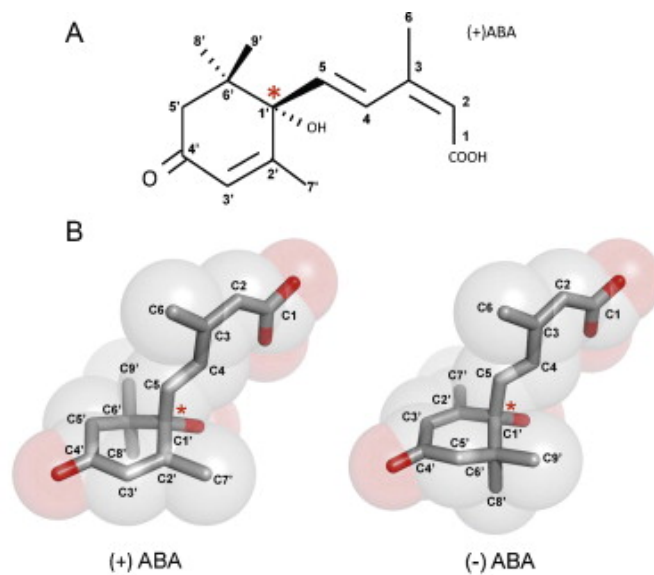
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## FIGURES

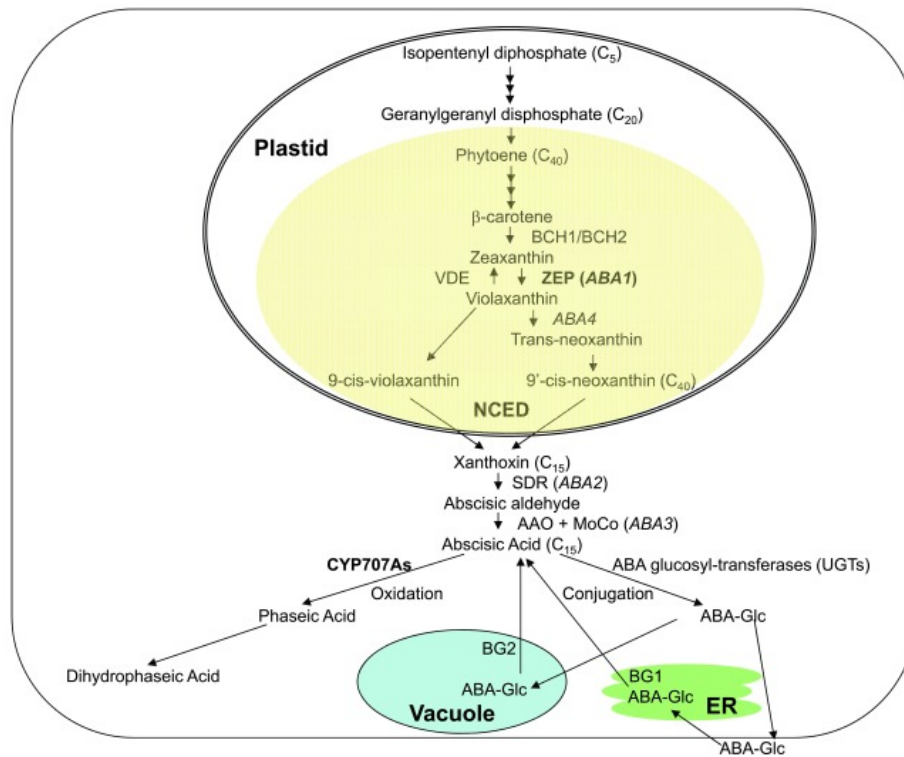


**Figure 1. Chemical structure of phytohormone ABA.**

**(A)** 2D structure of the natural S (+) form of ABA. The red asterisk points out the C1' asymmetric carbon of the molecule.

**(B)** 3D structure of the two ABA enantiomers. The (-) enantiomer has been rotated to illustrate the structural difference of the ring methyl groups with respect to the (+) enantiomer.

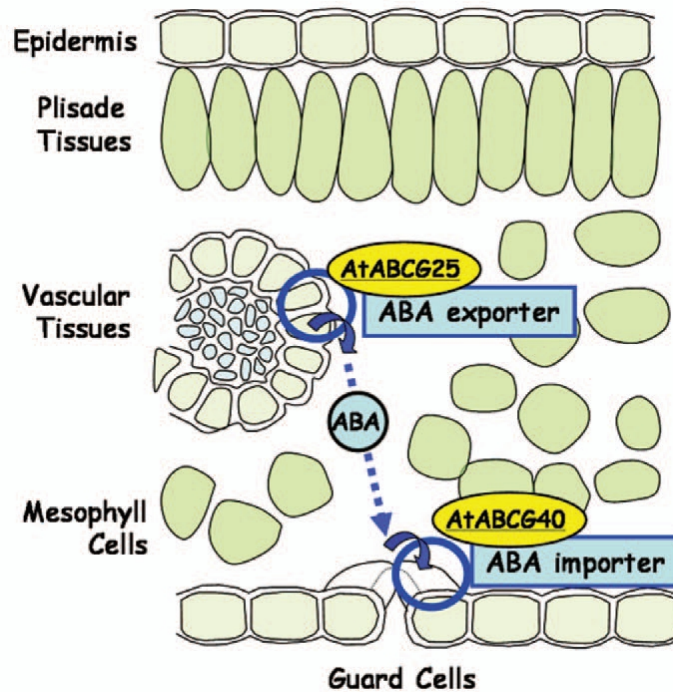
(Santiago, 2012)



**Figure 2. Pathways of ABA metabolism.**

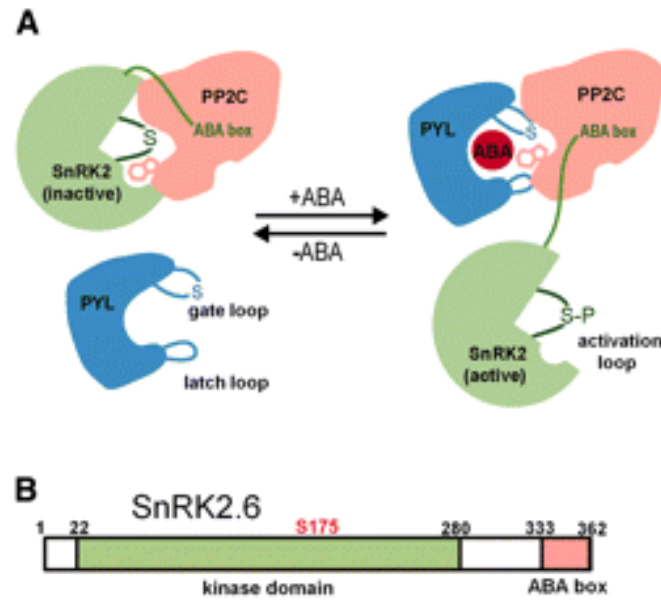
ABA biosynthesis, conjugation, and degradation pathways are indicated with key enzymes and subcellular localization of where these pathways are occurring.

(Finkelstein, 2013)



**Figure 3. Model of intercellular ABA signaling transmission via ABA transporters.**

This diagram is an Arabidopsis leaf section showing two distinct cell types: vascular tissues including vascular parenchyma cells and guard cells on the leaf epidermis. The current model is: AtABCG25 could function as an ABA exporter in ABA-biosynthesizing cells in the vasculature, transporting ABA out of the cell. AtABCG40 in the guard cells could then function as an ABA importer, transporting ABA from outside to inside of the guard cells to promote stomatal closure. (Kuromori, 2010)



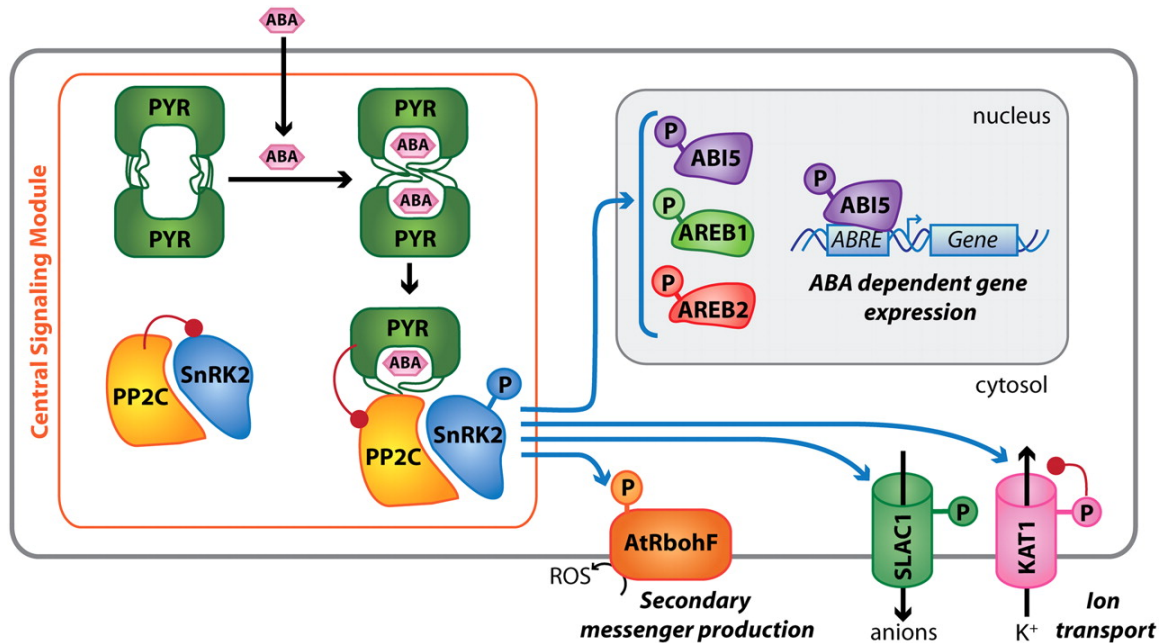
**Figure 4. Molecular mimicry regulates ABA signaling by SnRK2 and PP2C interactions.**

**(A)** Summary model for the interactions between SnRK2, PP2C, and the ABA receptor

PYR/PYL/RCAR in ABA signaling. In the absence of ABA, PP2C binds to the SnRK2 kinase domain and inhibits the kinase activity by dephosphorylating the activation loop serine and blocking the catalytic cleft. In the presence of ABA, ABA-receptor complex binds to PP2C and inhibits PP2C's catalytic activity by inserting the gate loop into the PP2C active cleft. PYL-mediated inhibition of PP2C allows activation of the SnRK2 kinase by activation loop autophosphorylation. The activated SnRK2 kinase then transmits the ABA signal by phosphorylating downstream factors.

**(B)** Schematic presentation of the domain structure of SnRK2.6 with key amino acid residue numbers shown on top.

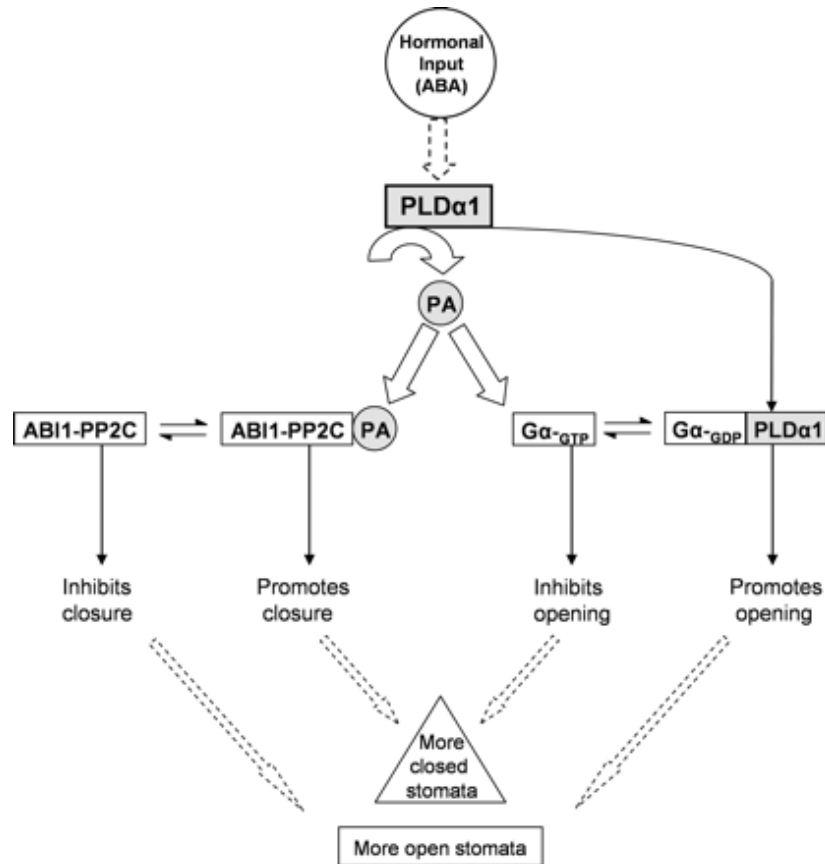
(Soon, 2012)



**Figure 5. The core ABA signaling components and downstream signaling pathways.**

Recent progress in understanding early ABA signal transduction has led to the construction of a PYR/RCAR–PP2C–SnRK2 signal transduction model. In the absence of ABA, PP2Cs inhibit protein kinase (SnRK2) activity through removal of activating phosphates (red connection on PP2C and SnRK2 indicates an inhibitory interaction). When ABA is present, ABA is bound by intracellular PYR/PYL dimers, which dissociate to form ABA receptor–PP2C complexes. Complex formation therefore inhibits the activity of the PP2C in an ABA-dependent manner (red connection between PYR and PP2C), allowing activation of SnRK2s. Several SnRK2 targets have been identified both at the plasma membrane and in the nucleus, resulting in control of ion channels, secondary messenger production, and gene expression.

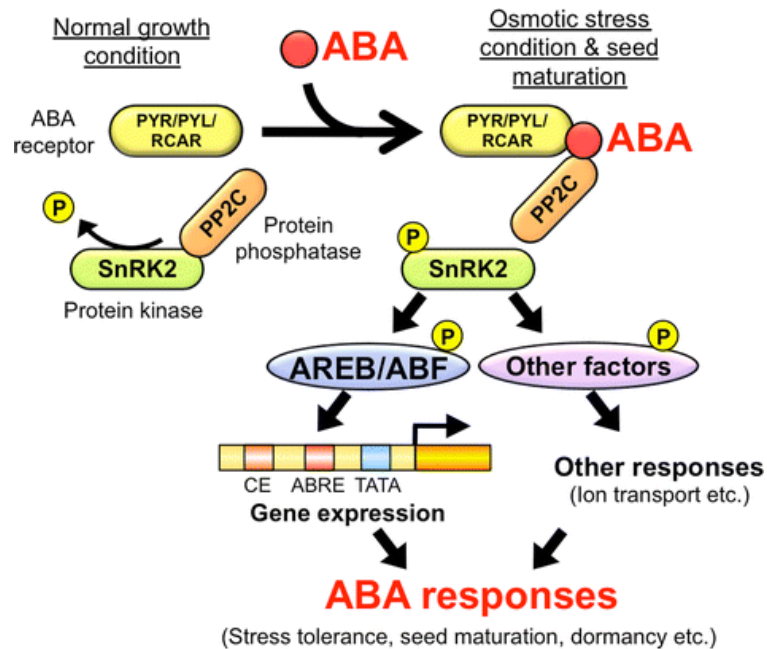
(Hubbard, 2010)



**Figure 6. Bifurcating pathway of ABA-mediated stomatal closure and opening.**

This model shows interaction among Phospholipase D (PLD $\alpha$ 1), Phosphatidic acid (PA), PP2C ABI1, and G PROTEIN  $\alpha$  SUBUNIT 1 (GPA1, G $\alpha$ ) in mediating ABA effects on stomatal closure and opening. PLD $\alpha$ 1-produced PA binds to ABI1, and this binding removes ABI1 inhibition of ABA promotion of stomatal closure. During ABA inhibition of stomatal opening, PLD $\alpha$ 1-produced PA acts upstream of GTP-bound G $\alpha$  (G $\alpha$ -GTP) to inhibit stomatal opening, whereas GDP-bound G $\alpha$  (G $\alpha$ -GDP) binds to PLD $\alpha$ 1 to suppress PLD activity. (Mishra, 2006).



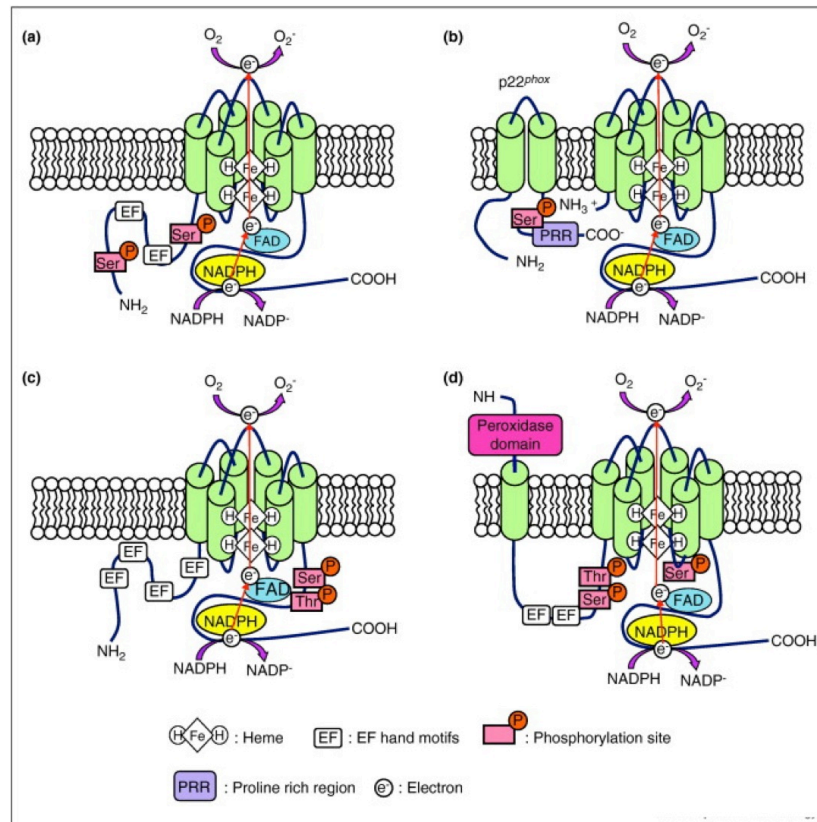


**Figure 7. The core ABA signaling pathway.**

Current model of the major ABA signaling pathway is: under normal growth conditions, PP2C negatively regulates SnRK2 kinase activity by direct interactions and dephosphorylation. When endogenous ABA is up-regulated by abiotic stresses, ABA receptors PYR/PYL/RCAR binds ABA and interacts with PP2C to inhibit protein phosphatase activity. This interaction releases SnRK2 from PP2C-dependent inhibition and activates SnRK2 kinase activity. Then SnRK2 can phosphorylate downstream factors including the AREB/ABF TFs and other factors (such as membrane proteins involving ion channels), to induce stress-responsive gene expression, which leads to ABA-induced stress responses.

ABRE, ABA-responsive element; AREB, ABRE-binding protein; ABF, ABRE-binding factor.

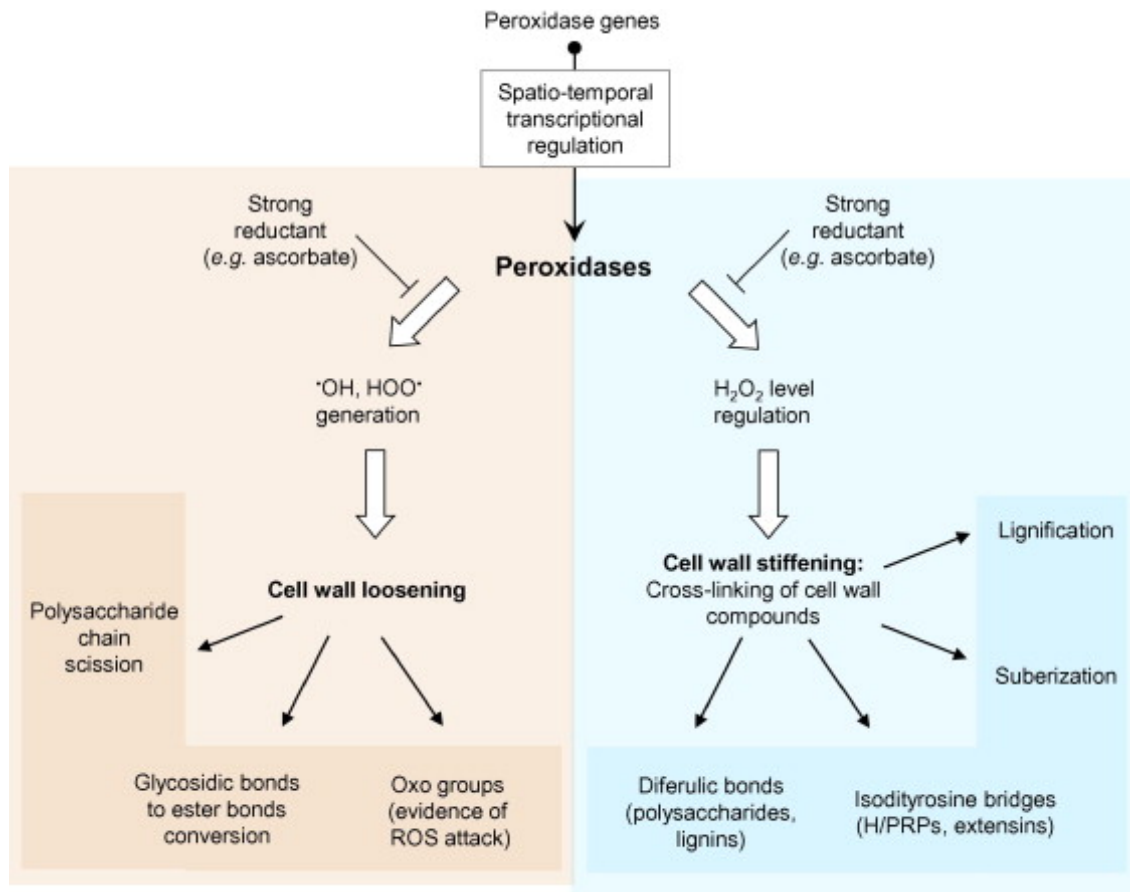
(Nakashima, 2013)



**Figure 8. Structure of NADPH oxidases in plants and mammals.**

A conserved C-terminal core region consisting of 6 transmembrane  $\alpha$ -helices (cylinders) and 2 heme groups (indicated by 'H' and 'Fe') is found in all RBOHs. **(a)** Plant RBOH; Two EF hand motifs are present in the N-terminal region. **(b)** Mammalian Nox1-Nox4; This Nox subfamily forms heterodimer with  $p22^{phox}$  that contains 2 transmembrane  $\alpha$ -helices and a proline rich region (PRR). **(c)** Mammalian Nox5; Four EF hand motifs are present in the N-terminal region. **(d)** Mammalian Duox; Peroxidase domain in the N-terminal region is involved in  $H_2O_2$  generation.

(Suzuki, 2011)

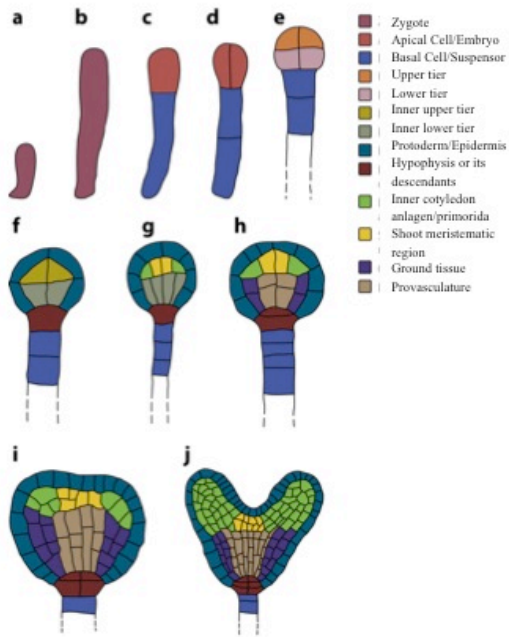


**Figure 9. Class III peroxidases can modify cell wall via two different pathways.**

Cell wall peroxidases (Prx) have dual activities in cell wall modifications: Class III Prxs are capable of generating reactive oxygen species (ROS) such as hydroxyl radicals ( $\text{HO}^\cdot$ ), but can also regulate the level of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Therefore, they play an important role in cellular growth by controlling the subtle balance between cell wall loosening (left part of the figure) and *de novo* cell wall synthesis/cell wall strengthening (right part of the figure).

H/PRPs = Hydroxyproline/Proline-Rich Proteins.

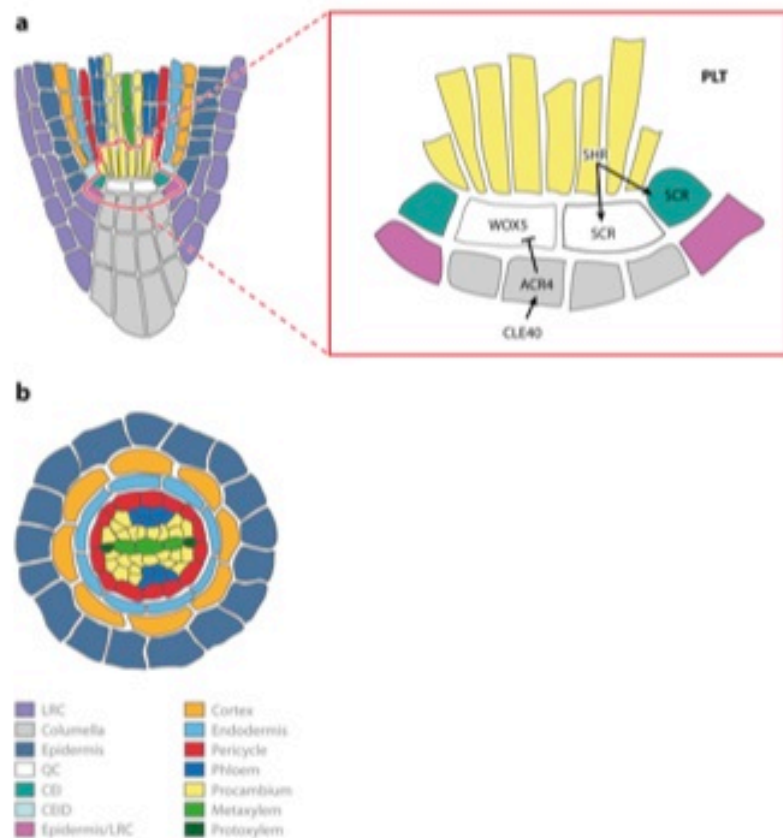
(Francoz, 2014)



**Figure 10. Early embryogenesis in Arabidopsis.**

Panels show longitudinal sections of embryos during consecutive developmental stages: (a) zygote, (b) elongated zygote, (c) one-cell stage, (d) two- or four-cell stage, (e) octant stage, (f) dermatogen stage, (g) early-globular stage, (h) mid-globular stage, (i) transition stage, and (j) heart stage. Groups of developmentally related cells are color-coded.

(Lau, 2012)

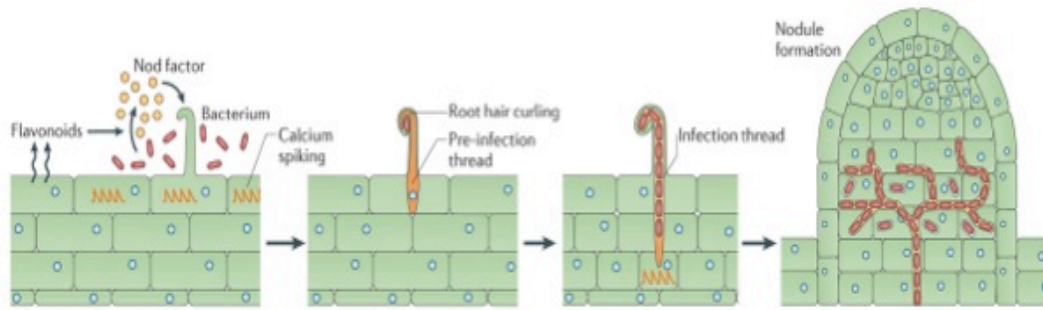


**Figure 11. Root apical meristem patterning in *Arabidopsis* root.**

(a) Organization of the root apical meristem. Different cell types are arranged in cell files along the length of the root. The magnified region shows the stem cell niche and regulatory interactions that maintain it. SHORTROOT (SHR) expressed in the stele moves into the quiescent center (QC) and cortex/endodermal initial (CEI) cells to maintain QC and stem cell identity; WOX5 maintains identity of the surrounding stem cells. WOX5 expression is confined to the QC through repression by ACR4, triggered by the CLE40 signal originating from differentiating columella cells. PLT expression throughout the niche also maintains QC and stem cell identity.

**(b)** Organization of cell types within the stele. The diagram shows a cross section of the root tip. The pattern of cell types in the stele is bilaterally symmetric: a central axis of xylem is flanked by two phloem bundles.

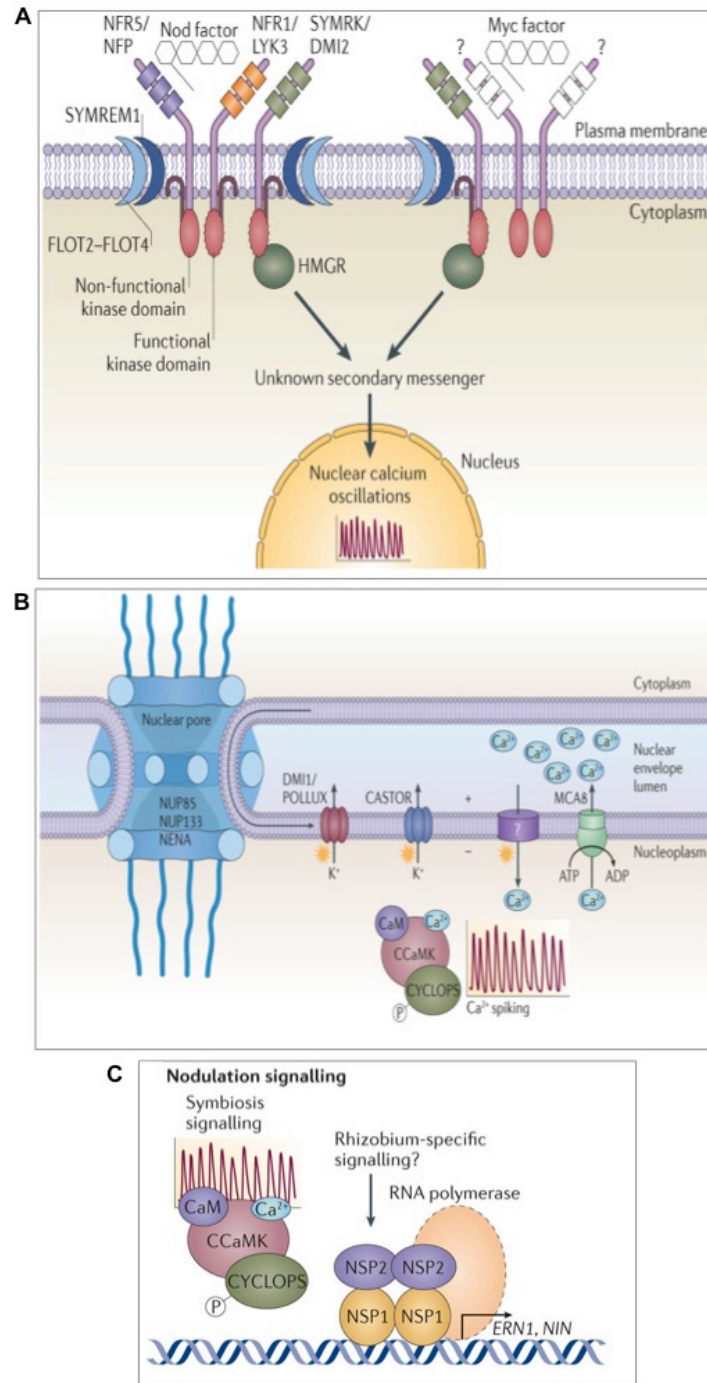
(Petricka, 2012)



**Figure 12. Rhizobia infection in *M. truncatula* roots.**

Initial signal exchange between legume and rhizobia is: flavonoids released by the plant root is perceived by rhizobia in the rhizosphere, which in turn produce nodulation factors (Nod factors) that are recognized by the plant. Nod factor perception activates the symbiotic signaling pathway, leading to calcium spiking. Rhizobia gain entry into the plant root by root hair cells that grow around the bacteria attached at the root surface, trapping the bacteria inside a root hair curl. Infection threads (ITs) are invasive invaginations of the plant cell; they are initiated at the site of root hair curls and allow invasion of the rhizobia into the root tissue. Nodules initiate below the site of bacterial infection, and are formed by *de novo* initiation of a nodule meristem in the root cortex. ITs grow towards the emerging nodules and ramify within the nodule tissue. Then bacteria are released into membrane-bound compartments inside the cells of the nodule, where the bacteria can differentiate into a nitrogen-fixing state.

(Oldroyd, 2013)



**Figure 13. Nodulation factor signaling pathway in *M. truncatula*.**

(A) Current model for Nod factor receptors. In *Lotus japonicus* and *Medicago truncatula* (*L. japonicus* protein names are given first, followed by *M. truncatula* protein names), Nod factor

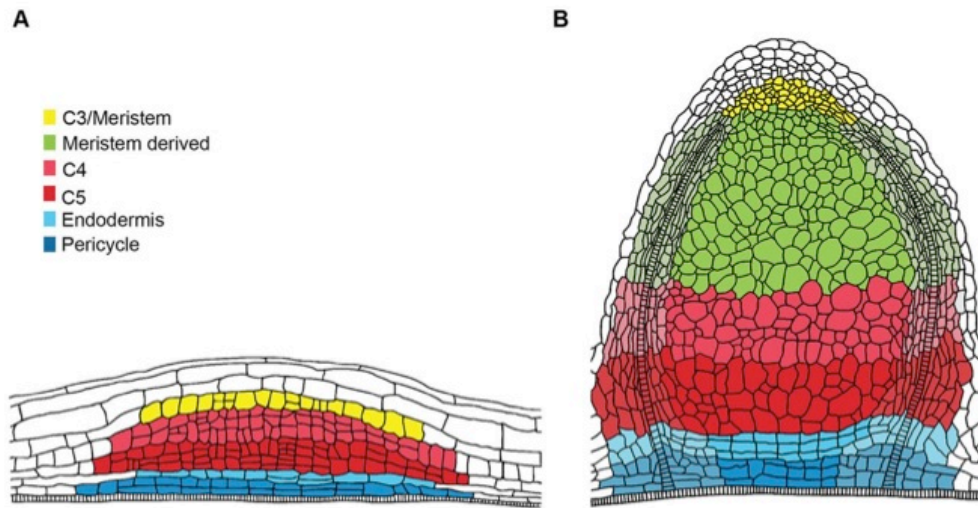


receptor 1 (NFR1)/LysM receptor kinase 3 (LYK3), NFR5/Nod factor perception (NFP) and Symbiosis Receptor-like Kinase (SYMRK)/DMI2 form a receptor complex associated with the recognition of Nod factor. The cytoplasmic domains of NFR1/LYK3 and SYMRK/DMI2 have functional kinase activity. SYMRK/DMI2 also has a role in mycorrhizal signalling and is proposed to associate with as-yet-unknown mycorrhizal factor (Myc factor) receptors.

(B) Current understanding of the symbiotic machinery for calcium oscillations. Downstream of the symbiotic receptors, POLLUX (in *Lotus japonicus*) or DMI1 (in *Medicago truncatula*) and CASTOR (in *Lotus japonicus*) are responsible for the generation of calcium oscillations, all of which are associated with the nuclear membranes.

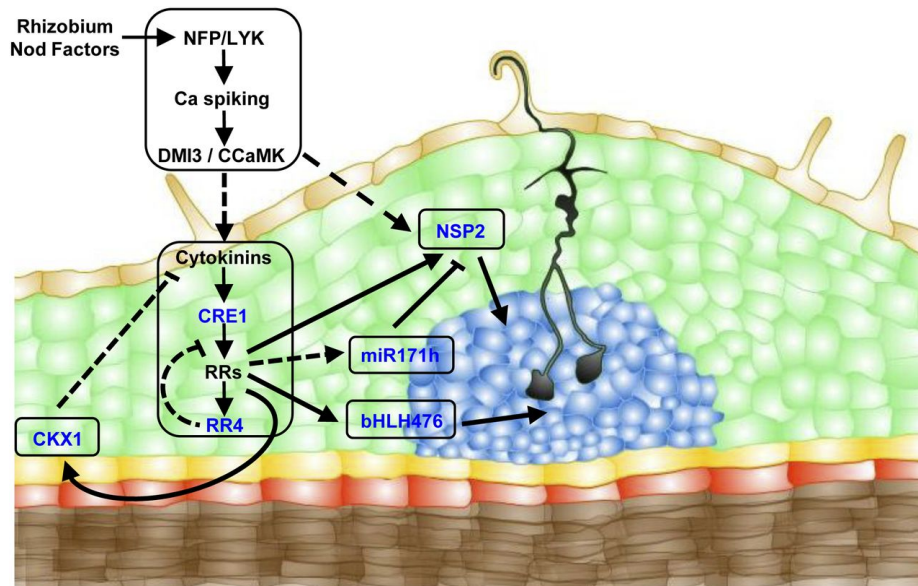
(C) Symbiotic signaling downstream of calcium oscillations. Calcium- and calmodulin-dependent serine/threonine protein kinase (CCaMK) is responsible for decoding the calcium oscillations, through its association with calcium and calmodulin (CaM). CCaMK associates with and phosphorylates CYCLOPS. Transcription factors Nodulation Signaling Pathway 1 (NSP1) and NSP2 are required for rhizobium-specific gene expression and associate with the promoters of rhizobium-induced genes, such as Nodule Inception (*NIN*) and Ethylene Response Factor Required for Nodulation 1 (*ERN1*).

(Oldroyd, 2013)



**Figure 14. Indeterminate root nodule fate map of *M. truncatula*.**

The origins of cells in nodule primordium (A) and nodule (B) are indicated by the same color. Note the meristem in a mature nodule (B) is originated from the third layer of cortex (C3) in (A). In a mature nodule, about eight cell layers of the basal part of the nodule tissue are derived from the fourth and fifth layers of cortex (C4 and C5), and not from the meristem. (Xiao, 2014)



**Figure 15. Cytokinin signaling in symbiotic nodulation.**

In the epidermis, upon the perception of rhizobium Nod factors by NFP/LYK receptors in *M. truncatula*, calcium spiking is induced and decoded by DMI1/CCaMK, which then triggers downstream gene expression changes via NSP2. In the cortex, cytokinin signaling is activated in response to rhizobia, and is transduced by cytokinin receptor CRE1 and Type-B RR1. Two direct targets of RR1 have been identified: Type-A RR4 and NSP2. A microRNA miR171h, which targets NSP2, and a cytokinin oxidase CKX1, as well as a TF bHLH476, are also potential downstream targets of RR1.

NFP/LYK, Nod Factor Perception/Lys-M Kinase receptor; DMI3/CCaMK, Does Not Make Infections/Calcium Calmodulin Protein Kinase; NSP2, Nod factor Signaling Pathway 2 the CRE1, Cytokinin Response 1; RR, Response Regulators.

Orange indicates epidermis, green indicates cortex, yellow indicates endodermis, red indicates pericycle, brown indicates stele, and blue indicates nodule primordium.

(Ariel, 2012)

**CHAPTER TWO: ABSCISIC ACID AND LATERAL ROOT ORGAN  
DEFECTIVE/NUMEROUS INFECTIONS AND POLYPHENOLICS MODULATE ROOT  
ELONGATION VIA REACTIVE OXYGEN SPECIES IN *MEDICAGO TRUNCATULA***

## ABSTRACT

Abscisic Acid (ABA) modulates root growth in plants grown under normal and stress conditions and can rescue the root growth defects of the *Medicago truncatula* lateral root-organ defective (*latd*) mutant. Here we demonstrate that Reactive Oxygen Species (ROS) function downstream of ABA in the regulation of root growth by controlling cell elongation. We also show that the MtLATD/NIP nitrate transporter is required for ROS homeostasis and cell elongation in roots, and that this balance is perturbed in *latd* mutants, leading to an excess of superoxide and hydrogen peroxide and a corresponding decrease in cell elongation. We find that expression of the superoxide-generating NADPH oxidase genes, *MtRbohA* and *MtRbohC*, is increased in *latd* roots and that inhibition of NADPH oxidase activity pharmacologically can both reduce *latd* root ROS levels and increase cell length, implicating NADPH oxidase function in *latd* root growth defects. Finally, we demonstrate that ABA treatment alleviates ectopic ROS accumulation in *latd* roots, restores *MtRbohC* expression to wild-type levels and promotes an increase in cell length. Reducing expression of *MtRbohC* using RNA interference leads to increased root elongation in both wild-type and *latd* roots. These results reveal a mechanism by which the MtLATD/NIP nitrate transporter and ABA modulate root elongation via superoxide generation by the MtRbohC NADPH oxidase.

## INTRODUCTION

Absciscic acid (ABA) is a key regulator of both plant development and stress responses. ABA is often thought of as a growth inhibitor, negatively regulating aspects of plant development such as root elongation (Pilet, 1975) and seed germination (Finkelstein et al., 2002), but in certain stress conditions, such as drought, ABA can also play a positive role, promoting root elongation and maintaining root growth (Sharp et al., 2004). Recently, ABA has been shown to also have a positive effect on root growth under non-stressed conditions, indicating that ABA can also act as a growth stimulator (Cheng et al., 2002). ABA regulates root growth by maintaining the *Arabidopsis* root apical meristem, where it promotes the quiescence of the quiescent center (QC), as well as the local suppression of differentiation (Zhang et al., 2010). However, the role of ABA on cell elongation during root growth has not been investigated.

The *LATERAL ROOT ORGAN DEFECTIVE/NUMEROUS INFECTIONS AND POLYPHENOLICS* (*LATD/NIP*) gene, also known as NPF1.7, encodes a nitrate transporter of *Medicago truncatula* that is required for root and nodule meristem development (Veereshlingam et al., 2004; Bright et al., 2005; Liang et al., 2007; Yendrek et al., 2010; Bagchi et al., 2012; L  ran et al., 2014). In plants homozygous for the *latd* mutation, the strongest allele of the *LATD/NIP* locus, the root apical meristem (RAM) undergoes exhaustion as development proceeds, ultimately arresting by 21 days (Bright et al., 2005; Liang et al., 2007; Yendrek et al., 2010; Bagchi et al., 2012). *latd* mutants also exhibit severe defects in the elongation of primary and lateral roots and in the formation of symbiotic root nodules (Veereshlingam et al., 2004; Bright et al., 2005; Liang et al., 2007). Interestingly, application of ABA can maintain the viability of the *latd* RAM and prevent it from arresting (Liang et al., 2007). The mechanism by which ABA acts to rescue root elongation in *latd* mutants is unknown. *latd* mutants have normal

levels of ABA (Liang et al., 2007) so the defect is likely to be in transport or signaling. The rescue of *latd* root growth by ABA indicates a positive role for ABA in RAM function, but not the mechanism by which it regulates root elongation.

Root growth is a function both of cell division and cell elongation. Rapid root growth is due largely to rapid elongation of cells in the growing root. Cell division is restricted to the meristematic region at the root tip and is required for continued growth of the root. Newly formed cells at the edge of the meristem exit the cell cycle and begin the process of differentiation, first elongating, undergoing a rapid increase in size, then beginning to differentiate (Benfey et al., 2010). The transition between proliferative growth and differentiation in the root is controlled by the transcription factor UPBEAT1 (UBP1), which regulates Reactive Oxygen Species (ROS) homeostasis (Tsukagoshi et al., 2010).

ROS are important signaling molecules that affect response to environmental signals as well as many aspects of plant development. In Arabidopsis root development, ROS signaling is not only crucial for RAM organization and maintenance (De Tullio et al., 2010; Tsukagoshi et al., 2010), but also for root hair formation (Foreman et al., 2003). Disruption in ROS signaling leads to defects in these developmental processes. ROS are also integral parts of hormone signaling networks (Mittler et al., 2011), and play an important role in plant responses under stress conditions (Achard et al., 2006; Achard et al., 2008). In drought conditions, ABA induces stomatal closure by stimulating the production of the ROS molecule hydrogen peroxide ( $H_2O_2$ ) in guard cells (Zhang et al., 2001; Wang and Song, 2008; Jannat et al., 2011), via a pathway involving guard cell-specific NADPH oxidases (Kwak et al., 2003). Plasma membrane-localized NADPH oxidases, named RESPIRATORY BURST OXIDASE HOMOLOGS (Rboh) in plants, are enzymes that play a key regulatory role in the ROS signaling network, catalyzing the

production of extracellular superoxide ( $O_2^{\cdot-}$ ) from molecular oxygen (reviewed in (Suzuki et al., 2011). Subsequently, short-lived  $O_2^{\cdot-}$  can be converted by superoxide dismutases (SODs) to  $H_2O_2$ , which can signal both intra- and extracellularly (Alscher et al., 2002).

Because of their role in  $O_2^{\cdot-}$  production, Rboh genes play critical functions in processes modulating ROS homeostasis, both in stress responses and during development, including that of roots. In *Arabidopsis*, *AtRbohC/RHD2* is required for root hair tip growth (Foreman et al., 2003; Takeda et al., 2008) and *AtRbohF* is required for casparian strip formation (Lee et al., 2013) and functions with *AtRbohD* to regulate root length in response to ABA (Kwak et al., 2003). In *Phaseolus vulgaris*, *PvRbohB* promotes lateral root elongation (Montiel et al., 2012; Montiel et al., 2013) and the formation of symbiotic root nodules (Montiel et al., 2012; Arthikala et al., 2013). In *M. truncatula*, *MtRbohA* promotes nitrogen fixation in root nodules, but whether it functions in root growth has not been explored (Marino et al., 2011), and no other *MtRboh* genes have been functionally characterized.

Here we report that ROS functions downstream of ABA in the regulation of root growth by controlling cell elongation. We find that *latd* mutant roots have higher levels both of  $O_2^{\cdot-}$  and  $H_2O_2$  than wild-type, and that expression of two  $O_2^{\cdot-}$ -generating NADPH oxidase genes, *MtRbohA* and *MtRbohC*, is increased in *latd* mutant roots while that of the predicted apoplastic ROS-scavenging gene Cu/ZnSOD is reduced. Additionally, we demonstrate that ABA can rescue the ectopic ROS accumulation and cell elongation defects in *latd* mutant roots, as can decreasing Rboh function with the pharmacological inhibitor diphenylene iodonium (DPI). We find that ABA treatment reduces *MtRbohC* expression in *latd* roots to wild-type levels, and that inhibiting *MtRbohC* expression using RNA interference (RNAi) leads to increased root elongation and in both wild-type and *latd* mutants. In summary, we find that ABA functions as a positive growth



regulator stimulating cell elongation, and thus root elongation, via inhibition of RbohC-mediated ROS signaling, rescuing the cell elongation defect of mutants lacking MtLATD/NIP.

## RESULTS

### The Balance of ROS Levels is Disrupted in *latd* Mutant Roots

The phenotypes of *latd* mutants are pleiotropic, with defects in several aspects of root growth as well as in stomatal function (Bright et al., 2005; Liang et al., 2007). The reduced root length, as well as reduced root hair growth relative to wild-type, is reminiscent of the root hair phenotype of *AtrbohC/rhd2* mutants and the root growth phenotype of *AtrbohF* mutants (Foreman et al., 2003; Kwak et al., 2003; Takeda et al., 2008). The RAM arrest of *latd* mutants is reminiscent of that of *root meristemless (rml)* mutants, which are defective in the synthesis of glutathione, a central component in the regulation of cellular redox homeostasis (Cheng et al., 1995; Vernoux et al., 2000); however, glutathione treatment does not rescue *latd* root growth (Bright et al., 2005). To test whether *latd* mutant roots have altered ROS levels, we used the ROS-reactive dyes nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) to measure levels of the ROS molecules  $O_2^{\cdot -}$  and  $H_2O_2$ , respectively, in different parts of the root (Ramu et al., 2002; Fester and Hause, 2005). We found that wild-type roots had strong NBT staining in the tip, with the strongest staining in the first millimeter of primary roots, but only weak staining in the rest of the root (Fig. 1A). In wild-type roots, the intensity of NBT staining starts to decrease in the elongation zone (EZ) and differentiation zone (DZ), behind the root meristem (Fig. 1A), with only light staining observed at a distance of 10 mm from the root tip (Fig. 1C). In longitudinal

sections of the mature region of wild-type roots (taken approximately 5 mm from the tip), staining is found mostly in the cortex and endodermis, with almost none in the vasculature (Fig. 1E). Lateral roots have a similar  $O_2^-$  accumulation pattern, with the darkest staining in the tips (Supplemental Fig. S1). Given the pattern of NBT staining in wild-type, we focused on two locations in the roots: the first 3 mm of the root tip containing approximately 1 mm of meristem, and a region 10 mm away from the tip, where cells have finished elongation and are mature (Fig. 1N). In *latd* mutants, the root tips showed strong NBT staining in the root tip, as in wild-type, but unlike wild-type, strong staining was also detected in the elongation zone, the differentiation zone (Fig. 1B) and the mature region of the roots, even as far as 10 mm from the root tip (Fig. 1D) leading to an overall increase of  $O_2^-$  in the root (Fig. 1G). We observe a similar pattern in plants carrying the *nip-1* mutation, a weaker allele of the *LATD/NIP* locus (Veereshlingam et al., 2004; Yendrek et al., 2010) (Supplemental Figure S2). The strength of NBT staining in the *latd*, *nip-1* and *nip-3* mutants reflects an allelic series corresponding to the severity of root and nodule defects (Veereshlingam et al., 2004; Bright et al., 2005; Teillet et al., 2008; Yendrek et al., 2010) (Supplemental Fig. S2), although both *latd* and *nip-1* have similar nitrate transport defects (Bagchi et al., 2012). Longitudinal sections of *latd* mutant roots revealed that mature regions of the *latd* root (5 mm from the root tip) exhibit ectopic accumulation of  $O_2^-$ , especially in the inner cortex, endodermis, and the vascular bundle (Fig. 1F). In *latd* roots, this ectopic NBT staining is detected particularly in the crosswalls of cortical cells but not on the sides parallel to the main axis of the root (see arrowheads in Fig. 1F).

$H_2O_2$  detected by DAB is localized primarily in the root tip of wild-type plants (Fig. 1H). *latd* mutants also show root tip DAB staining (Fig. 1I); however, DAB staining starts to give a strong signal approximately two millimeters from the tip of *latd* roots in the differentiation zone (Fig. 1I, arrowhead), where wild-type plants exhibit only a very low signal (Fig. 1H). This increased  $H_2O_2$  accumulation in *latd* roots persists throughout the mature region of the root,

whereas wild-type roots display no significant DAB staining in these regions (Fig. 1J and K). When dihydrofluorescein diacetate (H<sub>2</sub>DCFDA) was used to detect intracellular H<sub>2</sub>O<sub>2</sub>, we also observed increased levels of H<sub>2</sub>O<sub>2</sub> in *latd* roots as compared to wild-type (Fig. 1L and M). Curiously, occasionally entire cells in the epidermis of *latd* mutant roots were lit uniformly with a strong H<sub>2</sub>DCFDA signal (Fig. 1M). Together, these data show that *latd* mutant roots have higher levels of both O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> than wild-type and that these are found ectopically in the elongation zone, the differentiation zone, and the mature region of the root.

### **Cell Elongation and Expansion are Severely Reduced in *latd* Mutant Roots**

*latd* mutants have short primary roots as well as lateral roots that fail to elongate (Liang et al., 2007). ROS are important signaling molecules that regulate root growth and root hair elongation (Foreman et al., 2003; Monshausen et al., 2007). We wondered whether the short root phenotype of *latd* mutants might be the result of defects in cell elongation. To test whether *latd* mutant roots have cell elongation defects, we measured epidermal cell length at 5 days, when *latd* mutant roots have cell elongation defects, we measured epidermal cell length at 5 days, when *latd* roots grow at a rate only slightly less than that of wild-type (Bright et al., 2005). To ensure that we were measuring comparable cells in both wild-type and *latd*, we measured cells at the same distance, 10 mm from the root tip in the mature zone, where root hair growth was complete. At this developmental stage, *latd* mutant roots still have organized cell files as wild-type plants do (Fig. 2A and B). We found that at the same distance from the root tip, *latd* mutant roots have significantly shortened epidermal cells that are nearly 40% shorter than wild-type (Fig. 2C). We also found that the width of *latd* root epidermal cells is significantly narrower than that of wild-type (Fig. 2D).

### **The Cell Elongation Defect in *latd* Mutant Roots Becomes Increasingly Severe as Meristem Arrest Progresses**

The meristem of *latd* roots undergoes exhaustion as development proceeds, and arrests completely by 3 weeks (Bright et al., 2005; Liang et al., 2007). We wondered whether cell elongation defects in *latd* mutant roots might also become more severe as development progressed. In order to test this, we quantified epidermal cell length in *latd* mutants at 21-day old, in which root growth had completely arrested. As described above, we measured cells 10 millimeters from the root tip, in the mature zone, so that we are measuring fully differentiated cells. In this way we could test whether cells that had recently completed differentiation at 5-day or at 21-day had differences in length. We found that at 21 days, cells at this position in *latd* mutant roots show more severe elongation defects, with epidermal cell length more than 50% shorter than wild-type and significantly narrower (Fig. 2E-H). Epidermal cell shape becomes more variable in *latd* mutant roots, varying from a standard rectangular shape by being more triangular, having pointed ends, or having more irregular shapes (Fig. 2F) Thus our data suggests that both the cell elongation defects and cell shape variability in *latd* mutant epidermal cells become more severe as the meristem arrests.

### **ABA Rescues Both the *latd* ROS and Cell Elongation Defect**

ABA can rescue the short root phenotype as well as lateral root elongation defects of *latd* mutants (Liang et al., 2007). Since *latd* roots have increased levels of ROS (Fig.1), we asked whether ABA might rescue *latd* root defects by decreasing the ROS levels. To test this hypothesis, we used NBT staining to detect  $O_2^-$  in wild-type plants and *latd* mutant roots, treated

with or without ABA for 5 days. We found that 10  $\mu$ M ABA decreases  $O_2^{\cdot -}$  levels in wild-type 3 mm root tips (Fig. 3A), the mature region (10 mm from the tip) (Fig. 3B and D), and overall  $O_2^{\cdot -}$  amount in the whole root (Fig. 3K). The same effect of ABA on  $O_2^{\cdot -}$  levels is also observed in wild-type lateral roots (Supplemental Fig. S1). At this concentration, ABA also decreases  $O_2^{\cdot -}$  levels in *latd* mutant root in both root regions (Fig. 3A, C and E). However, these ABA treatments did not obviously affect NBT staining in the meristem (approximately the first 1 mm of the root tips): the tips of both primary and lateral roots remain darkly stained with NBT in both wild-type and *latd* roots grown at both concentrations of ABA (Fig. 3F to I and Supplemental Fig. S1). Again we checked the roots of *nip-1*, an allele of *latd*, and we found ABA decreased  $O_2^{\cdot -}$  levels in *nip-1* roots in a similar manner (Supplemental Fig. S3). We also examined the effect of ABA on  $O_2^{\cdot -}$  levels in *latd* roots at a later developmental stage, at 21 days, when *latd* root growth arrests in plants grown in the absence of ABA (Liang et al., 2007). We found that continuous ABA treatment also decreases  $O_2^{\cdot -}$  levels in a dose-dependent manner at this stage (Fig. 3J). The similar effect of ABA on  $O_2^{\cdot -}$  levels in wild-type and *latd* may indicate that the response to ABA in *latd* is intact, but that the endogenous factors that control the ROS balance is disrupted.

To test whether exogenous ABA also decreases  $H_2O_2$  levels, we used DAB to stain for  $H_2O_2$  in ABA-treated roots. We found that a 5-day continuous 10  $\mu$ M ABA treatment did not significantly decrease DAB staining in wild-type roots (Supplemental Fig. S4A), indicating that ABA may regulate  $H_2O_2$  differently in roots than  $O_2^{\cdot -}$ , or alternatively ABA may regulate  $H_2O_2$  at a different time point. However, in the mature region of *latd* roots, where ABA-rescued lateral roots develop, ABA decreased  $H_2O_2$  accumulation, (Supplemental Fig. S4B and C). We found that, as the concentration of ABA applied increases, the lower boundary of  $H_2O_2$  accumulation in

the mature region of *latd* roots is shifted shootward, away from the root tip (arrowheads in Supplemental Fig. S4D and E).

We then tested whether ABA could also rescue the cell elongation defect in *latd* mutant roots. We measured epidermal cell length and width in wild-type and *latd* roots treated with 10  $\mu$ M ABA, and found that 10  $\mu$ M ABA increases both the length and width of epidermal cells in *latd* mutant roots but decreases them in wild-type (Fig. 4A and B). Since LATD/NIP transports nitrate (Bagchi et al., 2012), we also tested whether 10 mM nitrate could regulate ROS levels in either wild-type or *latd* mutant roots, but found no significant effect (data not shown). Thus, our findings indicate that ABA can rescue both ROS levels and cell elongation defects in *latd* mutant roots, and decrease  $O_2^-$  levels in wild-type roots, suggesting that ROS may function in the signaling pathway downstream of ABA in regulating root elongation.

### **Decreasing ROS Levels Can Increase Cell Elongation in *latd* Roots**

ROS molecules are important for regulating plant growth and development (Swanson and Gilroy, 2010). Thus, an imbalance of ROS levels can cause defects in root growth and differentiation. We wondered whether the high ROS levels found in *latd* mutants were an indirect effect of the *latd* mutation or rather a direct cause of the root elongation defects. To test this question, we directly manipulated ROS levels in the root and measured the length and width of newly mature epidermal cells as described above. To decrease  $O_2^-$  levels, we used either DPI to inhibit the activity of NADPH oxidases, which catalyze the production of  $O_2^-$ , or EUK 134 (SOD mimetic, SODm) to chemically mimic the activity of SODs (Rong et al., 1999), which scavenge  $O_2^-$ , converting it to  $H_2O_2$ . We found that after 2 days of DPI treatment, *latd* mutant roots

developed significantly longer epidermal cells (Fig. 5A). In contrast, the same DPI treatment decreases epidermal cell length in wild-type roots (Fig. 5A), suggesting that ROS levels must reach an optimal balance for normal root elongation, and that the increased ROS in *latd* roots is mediated by NADPH oxidase activity. This two-day DPI treatment is sufficient to reduce  $O_2^{\cdot -}$  accumulation in wild-type and *latd* roots, especially at the root tip and elongation zone (Fig. 5B). In addition, *latd* mutants treated with DPI or SODm showed an increase from 4.3% to 70.6% or 54.6%, respectively, in the percentage of plants that developed lateral roots longer than 2 mm (Fig. 5C and D). Together, these data indicate that decreasing ROS levels in *latd* mutant roots, either by inhibiting production or increasing scavenging, can increase cell elongation, and thus root elongation, in both primary and lateral roots, and that the increased ROS levels in *latd* roots is mediated, at least in part, by NADPH oxidase activity.

### **Regulation of *Rboh* Gene Expression by *LATD/NIP* and ABA**

Our data show that *latd* mutants have increased levels of root  $O_2^{\cdot -}$  (Fig. 1) and decreased length of epidermal cells (Fig. 2). Inhibiting the activity of NADPH oxidases with DPI decreases the level of  $O_2^{\cdot -}$  and partially restores the cell elongation defect (Fig. 5). This observation indicates that the excess ROS production in *latd* mutants is mediated by NADPH oxidases. The fact that DPI mimics the effect of ABA on *latd* root ROS levels, cell length and root elongation (Fig. 3 and 4) (Liang et al., 2007), suggests that NADPH oxidases may be a target of ABA signaling as well. The *Rboh* family comprises 10 genes in Arabidopsis and at least 7 in *M. truncatula* (Sagi and Fluhr, 2006; Lohar et al., 2007; Marino et al., 2011). Gene duplications occurred independently in the Brassicaceae and the Fabaceae, thus similar gene names do not indicate orthologous genes (Marino et al., 2011; Montiel et al., 2012). To determine which

*MtRboh* gene or genes were correlated with the *latd* root defects, we examined expression of these genes using qRT-PCR, to see whether the expression of any of these corresponded to *latd* root ROS levels and cell length phenotypes. We predicted that expression of the *MtRboh* gene responsible should be increased in *latd* mutants and decreased by ABA to approximately wild-type levels in *latd*, and further in wild-type, since this is the pattern of  $O_2^{\cdot -}$  staining that we observe (Fig. 1 and 3). We found that expression of *MtRbohA* and *MtRbohC* was increased in *latd* roots while that of *MtRbohD* was decreased (Fig. 6A and B). Several of the *MtRboh* genes were ABA-responsive, with *MtRbohA*, *MtRbohB* and *MtRbohF* expression increased by ABA and *MtRbohC*, *MtRbohG* expression decreased (Fig. 6A and B). However, only *RbohC* expression fulfilled our predictions, namely, that expression is increased in *latd* over wild-type and ABA treatments that rescue the *latd* root ROS and cell length defects (continuous treatment with 10  $\mu$ M ABA) reduce expression of *RbohC* to wild-type levels in *latd* and further in wild-type roots (Fig. 6A), suggesting that *RbohC* is likely the NADPH oxidase responsible for *latd* root ROS and cell elongation defects. The regulation of *RbohC* by ABA in both genetic backgrounds suggests that *RbohC* may also mediate ABA's effect on root ROS and cell length in wild-type roots as well as those of *latd* mutants.

#### **Other ROS-Related Genes are Also Regulated by *LATD/NIP* and ABA**

ROS are dynamic signaling molecules whose levels are under tight control, as the result of a balance between production and scavenging (Mittler et al., 2011). We wondered whether the high  $O_2^{\cdot -}$  and  $H_2O_2$  levels in *latd* mutant roots (Fig. 1) might be caused not just by increased expression of  $O_2^{\cdot -}$ -generating *Rboh* genes (Fig. 6A and B), but also by decreased expression of ROS-scavenging enzymes.



To further examine the regulation of the ROS-scavenging system in *latd* mutants, we examined the expression of SOD genes as well as peroxidases and other antioxidant genes. SOD enzymes act directly on  $O_2^-$ , so we examined the expression of several SOD genes in both wild-type and *latd* plants. Since plants have multiple SOD proteins targeted to different subcellular regions, we used BLAST to identify genes for putative cell wall-localized, chloroplast-localized and cytoplasmic-localized SODs. The cotton *Cu/ZnSOD*, *GhCSD3*, is known to be targeted to the cell wall (Kim et al., 2008). We searched for an *M. truncatula* gene with high homology and found that TC183733 has 81% amino acid identity to *GhCSD3*. This putative apoplasmic *Cu/ZnSOD* (*ApoCu/ZnSOD*, Medtr6g029200) has slightly but significantly decreased expression in *latd* mutant roots (Fig. 6C). ABA can increase this *Cu/ZnSOD* expression in wild-type roots, and also in *latd* roots to restore its expression to wild-type levels (Fig. 6C), the inverse of the *RbohC* expression pattern. On the other hand, an *FeSOD* gene (Genbank accession AFK34552.1; Medtr1g048990) that has 95% identity to a *Medicago sativa* *FeSOD* (Genbank accession AAL32441.1), predicted to be localized to the chloroplast (Alscher et al., 2002; Rubio et al., 2004; Asensio et al., 2012), as well as a *Cu/ZnSOD* (*CytoCu/ZnSOD*, Medtr7g114240) predicted to be cytoplasmic (Macovei et al., 2011), did not show a difference in gene expression between *latd* and wild-type roots (Supplemental Figure S5).

Because many of the *LATD*-regulated ROS genes we had identified are associated with cell wall ROS production (three *MtRboh* genes and the putatively cell wall-localized *apoCu/ZnSOD*), we asked whether other putatively cell wall-localized ROS enzymes might be regulated by *LATD*. Peroxidases have been shown to catalyze cell wall cross-linking in response to various environmental stimuli (Almagro et al., 2009). We examined a putative cell wall peroxidase, *cwPRX2* (Genbank AES66966.1; Medtr2g084000), with high expression levels in

roots according to the *M. truncatula* Gene Expression Atlas (Benedito et al., 2008; He et al., 2009) and with 82% amino acid identity to the cell-wall localized *PsPOX11* peroxidase from *Pisum sativum* (Kawahara, 2006). We found that the expression of *cwPRX2*, was significantly upregulated in *latd* mutant roots, and up-regulated by ABA only in *latd* mutants (Fig. 6D). Another peroxidase, *Rhizobium-Induced Peroxidase (RIP1)*, Medtr5g074860), which is expressed in roots, but induced to higher levels by nodulation and H<sub>2</sub>O<sub>2</sub> (Ramu et al., 2002), has decreased expression in *latd* roots, and ABA can stimulate its expression in a pattern similar to that of *apoCu/ZnSOD*, although *RIP1* expression is more strongly affected by the *latd* mutation (Fig. 6E). Finally, we checked the expression of some antioxidant genes that are known to be altered in *M. truncatula* in response to drought stress (Filippou et al., 2011). We found that expression of the drought-stress responsive genes, *Alternative Oxidase (AOX)*, Medtr5g026620) and *Glutathione S-Transferase (GST)*, Medtr1g026140), were also altered in *latd* roots, but not expression of cytosolic *Ascorbate Peroxidase (cAPX)*, Medtr4g061140) (Supplemental Figure S6) (Filippou et al., 2011).

In summary, *latd* mutant roots exhibit altered expression levels of many ROS-related genes, for both ROS-generating and scavenging systems, and many of these are also regulated by ABA. Regulation of *RbohC* and apoplastic Cu/ZnSOD by *latd* and ABA correlates with root O<sub>2</sub><sup>-</sup> levels: the presence of the *latd* mutation increases *RbohC* and decreases apoplastic *Cu/ZnSOD* expression and ABA treatment reverses this effect, returning expression to wild-type levels, suggesting that *RbohC* and apoplastic *Cu/ZnSOD* expression may be important targets of ABA-LATD/NIP signaling.

## Silencing *MtRbohC* Stimulates Root Elongation and Lateral Root Formation

*MtRbohC* root expression correlates both with root length and with ROS levels: when *RbohC* expression is high (i.e. in untreated *latd* roots), roots have ectopic ROS accumulation and root length is short; when *RbohC* expression in *latd* roots is decreased by ABA treatment to wild-type untreated levels (Fig. 6A), ROS levels decrease and roots are longer. In order to test whether the decrease in *RbohC* expression is the cause of increased root length in ABA-treated *latd* roots, we used RNA interference (RNAi) to reduce *RbohC* expression using *Agrobacterium rhizogenes*-mediated transformation. We confirmed that our RNAi approach efficiently reduced *RbohC* expression at least 5-fold, yet did not affect expression of *RbohD*, the most closely related *MtRboh* gene (Marino et al., 2011), or two other *MtRboh* genes, *RbohB* or *RbohA*, with strong root expression (Fig. 7H). We observed root elongation in *RbohC* RNAi transformed roots for 10 days. Our results showed that in wild-type plants, roots transformed with the *MtRbohC* RNAi construct have longer primary roots and an increased lateral root density (Fig. 7B and C). *latd* roots transformed with *RbohC* RNAi constructs showed similar results in both root elongation and lateral root density, although the latter one showed a big variance among four biological replicates leading to a non-statistical difference (Fig. 7D and E). We think this is due to the variability in the nature of *A. rhizogenes* transformation, but *latd* roots transformed with *MtRbohC* RNAi vector showed increased lateral root density in three out of four biological replicates. Although decreasing *RbohC* expression in *latd* mutants did not show full rescue of its root defects, the percentage of roots that developed lateral roots longer than 2 mm increased to at least 80% from about 30% (Figure 7G). Together, these findings indicate that down-regulation of *MtRbohC* expression using RNAi increases root elongation and lateral root formation in both wild-type and *latd* mutants, demonstrating an important role for *MtRbohC* as a negative regulator of root elongation.

## DISCUSSION

In this study we demonstrate that the MtLATD/NIP/MtNPF1.7 transporter is required for ROS homeostasis and cell elongation in roots. In *latd* mutants this balance is perturbed, leading to an excess of  $O_2^{\cdot -}$  and  $H_2O_2$  and a corresponding decrease in cell elongation (Fig. 1 and 2). We demonstrate that rescue of root growth by ABA in *latd* mutants is due to a decrease in ROS levels and an increase in cell elongation (Fig. 3 and 4). Directly manipulating ROS levels in *latd* mutant roots to reduce  $O_2^{\cdot -}$  levels can also increase cell length and promote root elongation, indicating that it is the change in root ROS levels that increases cell elongation in ABA-treated *latd* roots (Fig. 5). We propose that ABA can regulate cell elongation via manipulation of ROS levels in roots. Our data indicate that ABA reduces ROS levels and that this effect corresponds to changes in root length and cell length, suggesting that this is one mechanism by which ABA regulates root growth. The observation that the NADPH oxidase inhibitor, DPI, increases both cell and lateral root elongation in *latd* mutants indicates that *latd* mutant root elongation phenotype is likely mediated by NADPH oxidase activity (Fig. 5). We also find that expression of enzymes involved in ROS synthesis or degradation is regulated by ABA and LATD/NIP in a manner consistent with observed levels of ROS in roots (Fig. 6), suggesting transcriptional control of ROS-related enzymes by ABA and LATD/NIP. In particular, expression of the NADPH oxidase-encoding gene, *MtRbohC*, increases in *latd* roots and is decreased by ABA treatment, just as  $O_2^{\cdot -}$  levels are (Fig. 6). Reducing expression of *MtRbohC* leads to significant increase in root elongation in both wild-type and *latd* mutant roots, indicating that *MtRbohC* is a negative regulator of root elongation and that its increased expression in *latd* roots is a major cause of the *latd* short root phenotype. The change in *MtRbohC* expression in *latd* roots and in response to ABA and its

correlation with root and cell length indicate that MtRbohC is a key target of LATD/NIP and ABA signaling in the root.

### **ABA Regulates Root Cell Elongation by Modulating ROS Levels**

ABA plays an important role in regulating root length, both in drought conditions and during normal growth (Sharp et al., 1994). In well-watered plants, low ABA levels caused by mutations in ABA biosynthetic genes reduce root length, as do high levels of ABA caused by addition of the hormone to the growth medium (Sharp et al., 1994; Cheng et al., 2002; Lin et al., 2007), suggesting that under normal growth conditions there is an optimal ABA level for root elongation, and that deviating above or below that level reduces the ability of the root to elongate. Root growth is driven by two interrelated processes: cell division and cell elongation. Root meristem length and cortical cell length in different *Arabidopsis* accessions is highly correlated, indicating the fundamental linkage between these two processes (Slovak et al., 2014). ABA has been shown to promote QC quiescence and suppress stem cell differentiation in the RAM, which indicates ABA's positive role in regulating root growth (Zhang et al., 2010). In maize plants, continued root elongation under conditions of low water potential is reduced in ABA-deficient mutants, and elongation can be restored by addition of ABA (Sharp et al., 2004). Here, we show that ABA stimulates root cell elongation in *latd* mutants (Fig. 4) at the same concentrations that restore *latd* root tip morphology and meristem function (Liang et al., 2007). Interestingly, this concentration of ABA has the opposite effect on wild-type roots, where it decreases epidermal cell elongation (Fig. 4). This apparent contradiction is resolved by a model in which *latd* root tips experience either a low concentration of ABA or reduced responsiveness to ABA. As a result, addition of ABA to *latd* roots would bring them closer to a more optimal concentration of ABA.

Wild-type roots, in contrast, already experience a concentration of ABA that is more optimal for root growth. Addition of ABA drives them farther from this optimum and root elongation decreases.

ABA signals via ROS in multiple plant species and tissues, but whether ABA stimulates or inhibits ROS production differs in different species. In *Arabidopsis* and maize, ABA stimulates  $O_2^-$  and  $H_2O_2$  production in guard cells, leaves and roots via NADPH oxidase activity (Guan et al., 2000; Jiang and Zhang, 2001, 2002; Kwak et al., 2003; He et al., 2012). In contrast, our findings indicate that ABA treatment reduces levels of  $O_2^-$  in both wild-type and *latd* mutant roots in *M. truncatula* (Fig. 3). The observation that addition of ABA increases ROS levels in *Arabidopsis*, but decreases it in *Medicago*, may be the reason for the opposite response of legume and non-legume roots to ABA in the elaboration of root architecture (Liang and Harris, 2005). Briefly, ABA treatment stimulates lateral root formation in *Medicago* and several other legume species, while simultaneously inhibiting primary root elongation, thus strongly increasing lateral root density. Conversely, ABA significantly decreases lateral root density in non-legumes from several different plant families, by inhibiting lateral root formation more strongly than it inhibits primary root elongation (Liang and Harris, 2005). The one exception is *Casuarina glauca*, a non-legume that forms a nitrogen-fixing symbiosis with an actinomycete, and which increases lateral root density in response to ABA just as most legumes tested do (Liang and Harris, 2005). Based on the observed diversity of root architecture patterns throughout the plant kingdom, it is not surprising that different plant species will have evolved different responsiveness to hormones or different hormone responses. Thus, our analysis of the ABA response pathway in *Medicago* roots may reveal insight into the mechanism by which different plant species elaborate different root architectures.

## **A LATD/NIP Signaling Pathway Interacts with ABA to Regulate MtRBOH Gene Expression**

Here we present evidence that the LATD/NIP nitrate transporter and the plant hormone, ABA, combine to regulate expression of multiple *Rboh* genes in the *Medicago* root. Two genes, *MtRbohA* and *MtRbohC*, are regulated by both ABA and LATD/NIP, but in the case of *RbohA* the effect of ABA and LATD/NIP are additive, and in the case of *RbohC*, they are antagonistic (Fig. 6). The expression of *RbohC* corresponds to the level of  $O_2^{\cdot -}$  observed in *latd* roots. Under conditions that raise expression of *RbohC*, ROS levels also rise and under conditions that lower *RbohC*, ROS levels lower (Figures 3 and 6). In addition, root and cell length corresponds well with the levels both of  $O_2^{\cdot -}$  and of *RbohC* expression (Fig. 4). Why might *RbohC*-mediated  $O_2^{\cdot -}$  production be more important than that produced by other *Rboh* gene products? *RbohC* is expressed at lower levels in the root than any of the other *Rboh* genes (Marino et al., 2011) and the expression of many other genes is also regulated by LATD/NIP and ABA, thus regulation of *RbohC* expression may initially seem an unlikely mechanism. However, the tight correlation of *RbohC* expression with ROS levels and cell length suggest otherwise. The low expression of *RbohC* in root tissue may indicate that it is expressed only in a subset of cells. Ubeda-Thomas and colleagues have shown that endodermal cell expansion is rate-limiting for elongation of the root as a whole, thus demonstrating that factors which affect a small subgroup of root cells may have effects at the level of the whole root (Ubeda-Tomas et al., 2008). Perhaps the location of *RbohC* activity in the root is critical to its function in controlling cell and root length. It will be interesting to see which cells express *RbohC* in both wild-type and *latd* mutants, and in this way add to our understanding of the control of root growth.

### **Could ABA-Regulated ROS Modulate Cell Elongation by Acting Directly on the Cell Wall?**

Many studies have shown that ROS can interact with plant cell wall components in order to regulate growth (Gapper and Dolan, 2006).  $\text{H}_2\text{O}_2$  has been shown to crosslink cell wall structural proteins during plant disease resistance in soybeans (Brisson et al., 1994), or induce cell wall crosslinking between cell wall polymers in maize coleoptiles, thus stiffening cell walls and limiting growth (Schopfer, 1996). Conversely, hydroxyl radicals can induce cell wall loosening, thus allowing cell expansion (Fry, 1998). In our study, we noticed that  $\text{O}_2^-$  accumulates ectopically at apical/basal cell junctions in *latd* mutant roots, which may lead to local cell wall stiffening (Fig. 1E). Although there is no direct evidence linking extracellular  $\text{O}_2^-$  with cell wall modification,  $\text{O}_2^-$  can be converted to  $\text{H}_2\text{O}_2$  very rapidly either through non-enzymatic reactions, or by enzymes such as SOD (Apel and Hirt, 2004). Thus it is possible that an increased level of  $\text{O}_2^-$  in the apoplast of *latd* mutant roots may cause cell wall cross-linking by generating more  $\text{H}_2\text{O}_2$  in the cell wall, and lead to premature growth cessation, and thus shorter cells.

We find that ABA can decrease ROS levels in *latd* mutant roots and promote cell elongation. Since ROS can interact with cell wall components to either loosen or strengthen the cell wall to regulate growth, we hypothesize that ABA may function to regulate cell elongation by altering cell wall ROS levels. Alternatively, ABA regulation of cell elongation could be via a separate pathway. In *M. truncatula* seeds, it has been shown that inhibition of germination by ABA is related to the reduced expression of genes involved in cell wall loosening and expansion (Gimeno-Gilles et al., 2009). It is likely that in order to restrict or promote growth, ABA may directly regulate cell wall stiffening or loosening by triggering changes in apoplastic ROS production and scavenging to modulate the balance of different ROS species in the cell wall.



## **The Nitrate Transporter MtLATD/NIP/MtNPF1.7 Regulates Root ROS Levels to Control Root Elongation**

Uneven distribution of nitrate in the soil can locally stimulate root branching, thus altering the overall architecture of the root system (Zhang and Forde, 1998). The ability of the plant to respond to an inconsistent and changing environment is a reflection of the plasticity of root development. In order for the local environment to modulate root architecture, the root must first sense the change in environment, then coordinate development, most likely via a hormone, and ultimately regulate cell division and cell elongation. The *LATD/NIP* gene (MtNPF1.7) encodes a member of the NPF (NRT1/PTR) family of hormone, nitrate and di- or tri-peptide transporters (Yendrek et al., 2010; L  ran et al., 2014) and plays an important role in root architecture (Bright et al., 2005). The LATD/NIP protein has recently been shown to mediate transport of nitrate into cells (Bagchi et al., 2012) and is required for both lateral root elongation as well as primary root growth (Bright et al., 2005). An attractive model is that LATD/NIP may function to coordinate root elongation with nitrate sensing, although its role in nitrate sensing is still unknown.

Loss of LATD/NIP function results in complete loss of lateral root elongation: lateral roots arrest immediately after emergence from the primary root (Bright et al., 2005). This block to lateral root elongation can be bypassed by adding ABA to the growth medium (Liang et al., 2007). ABA levels in whole *latd* mutant seedlings are indistinguishable from that of wild-type (Liang et al., 2007), thus LATD/NIP must function after ABA synthesis. Since the *latd* root development defect can be rescued by adding ABA to the growth medium, the likeliest possibility is that *latd* mutants can produce ABA, but are unable to transport it to the needed location. The fact that this problem can be circumvented by providing ABA in the medium suggests that the medium is in

contact with the responding tissue, perhaps the root tip or epidermis. Alternatively, ABA and LATD/NIP could function in parallel, to independently regulate both ROS levels and root elongation by different mechanisms. NRT1.1 (AtNPF6.3) transports both nitrate and auxin, thus directly linking nitrate sensing with auxin transport and thus control of root growth (Krouk et al., 2010). It is interesting to speculate that LATD/NIP could perform a similar role in sensing nitrate and regulating ABA transport, either directly or indirectly. Other NPF family members have recently been shown to transport ABA (Kanno et al., 2012), suggesting that the link between LATD/NIP and ABA transport could be direct. We are currently testing this possibility.

Our findings that functioning of the LATD/NIP transporter is required for ROS homeostasis, which regulates cell elongation in the developing root, reveal a key role for LATD/NIP in control of root growth, and suggest a possible link to soil nitrate. Our data demonstrate that together, LATD/NIP, in concert with ABA, control expression of the  $O_2^{\cdot-}$ -generating RbohC enzyme in the root in a way that corresponds to  $O_2^{\cdot-}$  levels and to cell length. We do not yet know whether the effect of ROS and LATD/NIP on cell elongation is mediated by direct changes in cell wall loosening or rigidity or indirectly via cytoplasmic changes, perhaps to the cytoskeleton. The severe root defects caused by the loss of LATD/NIP function, despite the existence of several very close Medicago homologs, indicate an essential role for this nitrate transporter in root development via maintenance of ROS homeostasis, and suggest an intimate relationship between LATD/NIP function and ABA signaling.

## **MATERIALS AND METHODS**

## Plant Growth Conditions

*M. truncatula* seeds were scarified with concentrated sulfuric acid for 10 minutes, rinsed 6 times with sterile water, and sterilized in 30% Clorox, before imbibing for 5 to 6 hours, shaking at room temperature. Seeds were cold-treated for at least 24 hours before germinating in a moistened, sealed petri plate overnight in the dark. The A17 line was used as the wild-type control in all experiments. Seedlings were grown on 25 cm x 25 cm petri dishes (Nunc, <http://www.nuncbrand.com/>) or in growth pouches (<http://www.mega-international.com/>) containing buffered nodulation medium (BNM) at pH 6.5 (Ehrhardt et al., 1992) and placed vertically in an MTR30 Conviron growth chamber at 20°C, 50% humidity, 16 h light/8 h dark cycle with an intensity of 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ . Growth pouches were placed in a sealed Styrofoam box with a clear plastic dome as a lid. For ABA treatment, ( $\pm$ )-ABA (A1049; Sigma, <http://www.sigmaaldrich.com/>) was added to the medium after autoclaving to reach specified concentrations. Diphenylene iodonium (DPI, Sigma-Aldrich) or EUK 134 (Caymen Chemicals) were dissolved in DMSO and added to liquid medium in growth pouches to reach specified concentrations.

## ROS Staining

For  $\text{O}_2^-$  staining, nitroblue tetrazolium (NBT, Sigma-Aldrich) was used as described in Ramu et al. (Ramu et al., 2002) with modifications. Roots were incubated in 10 mM sodium phosphate buffer (pH 7.8) with 10 mM  $\text{NaN}_3$  and 1 mg/ml NBT for 30 minutes at 37 °C. The reaction was stopped by removing the NBT staining solution and washing roots twice in 80%

ethanol. All NBT staining was repeated four times for a total of at least 30 roots per condition. Whole-mount images were taken under a Leica dissecting microscope.

For longitudinal root sections, a minimum of five stained roots were cut into 1 cm segments and sectioned on a Lancer Vibratome Series 1000. All sections were 90  $\mu\text{m}$  thick. The images of sections were taken using an Olympus microscope.

For  $\text{O}_2^-$  quantification, NBT-stained roots were first ground in liquid  $\text{N}_2$  into a fine powder. Then the powder was dissolved in 2M KOH-DMSO (1/1.16) (v/v), followed by centrifugation at 12,000 g for 10 minutes. Absorbance at 630nm was immediately measured, then compared with a standard curve plotted from known amounts of NBT in the KOH-DMSO mix (Ramel et al., 2009). Experiments were from 3 biological replicates for a total of 9 to 28 roots per condition and genotype.

For  $\text{H}_2\text{O}_2$  staining, roots were incubated in 1 mg/ml 3,3'-diaminobenzidine (DAB, Sigma-Aldrich) in sodium citrate buffer at room temperature overnight, then washed and cleared with 10% lactic acid (Fester and Hause, 2005). Stained roots were imaged under a Leica dissecting microscope. For an alternative  $\text{H}_2\text{O}_2$  probe, 25  $\mu\text{M}$  dihydrofluorescein diacetate ( $\text{H}_2\text{DCFDA}$ , Sigma-Aldrich) in sodium phosphate buffer, pH 7.0 was used to stain whole roots for 30 minutes. Stained roots were then washed with fresh sodium phosphate buffer three times, and imaged under a Zeiss LSM 510 confocal microscope using the green emission filter. DAB staining was repeated three times for a total of 20 roots per condition.

## **Cell Length Measurement and Confocal Microscopy**

To measure cell length, whole roots were first stained with propidium iodide (Sigma-Aldrich) followed by washing in sterile water twice, then imaged under a Zeiss LSM 510 confocal microscope. Epidermal cell length was averaged from at least 10 cells per root at a distance of 1 cm from the root tips, from at least 5 roots examined for each treatment. Cell length was measured using Zeiss LSM 510 software.

## **Quantitative RT-PCR**

Total RNA was extracted from whole roots using RNeasy plant mini kits (Qiagen) following the manufacturer's protocol. Each treatment in all experiments was from 15 to 20 pooled roots; all experiments were repeated at least three times. RNA was quantified with a nanodrop then followed with DNase treatment (Turbo DNase-free kit, Ambion). RNA was subsequently cleaned and concentrated with Qiagen RNA Cleanup Kit. All RNAs were checked with an Agilent 2100 Bioanalyzer for good quality and integrity. cDNA was synthesized from one µg of total RNA using the SuperScript III first-strand synthesis system (Life Technologies, Invitrogen). qRT-PCR was performed using an ABI StepOnePlus Real-time PCR system (Applied Biosystems), using SYBR Green reagent (VWR). Data was collected with SDS 2.2 software (Applied Biosystems). Target gene relative expression was determined by normalizing with the geometric mean of the expression of two endogenous controls: UBC9 and PDF2 (Kakar et al., 2008). Statistical analysis was performed using one-way ANOVA in SPSS software (Version 20.0.0) on the relative gene expression from 3 biological replicates. Primers used in qRT-PCR were designed using Primer Express v3.0.1 software and are listed in Supplemental

Table S1.

### **RNAi Vector Construction and *Agrobacterium rhizogenes*-Mediated Transformation**

A 231-bp region of *MtRbohC* with low similarity to other *MtRboh* genes, was amplified from wild-type root cDNA (RbohC RNAi\_for, 5'-caccatgggaattgatgaaattgaaga-3'; RbohC RNAi\_rev, 5'-gccaaagcaaatcaagtttctc-3'). The purified PCR fragment was then cloned into the GATEWAY pENTR Directional TOPO Cloning kit (Invitrogen) following the manufacturer's instructions, and recombined into the destination vector pK7GWIWG2(II)-RedRoot (VIB Plant Systems Biology, University of Ghent) (Karimi et al., 2002; Op den Camp et al., 2011) to produce the RbohC RNAi vector, pCZ42. The pCZ42 vector was transformed into *Agrobacterium rhizogenes* strain Arqual (Quandt et al., 1993) and then used to transform both A17 and *latd* *Medicago truncatula* roots (Limpens et al., 2004). As a control, some plants of each genotype were transformed with a GUS RNAi construct. After two weeks, transgenic roots were identified by screening for DsRed fluorescence using a 525 nm long-pass emission filter on a Nikon TE200 microscope. All untransformed roots and all transformed roots but one were cut off the composite plants. Composite plants containing untransformed shoots and a single transformed root were then transferred to growth pouches containing BNM medium and placed in styrofoam boxes covered with a plastic dome and sealed with packing tape to maintain humidity. The boxes were placed in the growth chambers with settings as described above. Plants were grown for 10 days, and the medium was replenished once during this period. At 10 days, roots were examined under the fluorescence microscope again to confirm DsRed fluorescence before recording the final root growth phenotype. Experiments were done three times, for a total of 25 to 37 composite plants of each genotype/vector combination over the course of the four experiments.

### Accession numbers

The sequences for genes used in this article can be found under the following accession numbers: *MtRbohA* (Medtr1g083290), *MtRbohB* (Medtr3g098380), *MtRbohC* (Medtr3g098350), *MtRbohD* (Medtr3g098320), *MtRbohE* (Medtr8g095520), *MtRbohF* (Medtr7g060540), *MtRbohG* (Medtr7g113130), *ApoCu/ZnSOD* (Medtr6g029200), *CytoCu/ZnSOD* (Medtr7g114240), *cwPRX2* (Medtr2g084000), *cAPX* (Medtr4g061140), *GST* (Medtr1g026140), *AOX* (Medtr5g026620), *FeSOD* (Medtr1g048990), *RIP1* (Medtr5g074860), *UBC9* (Medtr7g116940), *PDF2* (Medtr6g084690).

### SUPPLEMENTAL MATERIALS

**Supplemental Figure S1.** Seven-day old wild-type lateral roots at different developmental stages stained for  $O_2^-$  with NBT.

**Supplemental Figure S2.** The *nip-1* allele of the *LATD/NIP* gene has increased superoxide ( $O_2^-$ ) levels.

**Supplemental Figure S3.** ABA decreases superoxide levels in *nip-1* roots, as it does for *latd* roots.

**Supplemental Figure S4.** DAB staining of  $H_2O_2$  in 5-day old wild-type and *latd* roots grown with and without 10  $\mu$ M ABA.

**Supplemental Figure S5.** Relative Expression of *FeSOD* and a cytoplasmic *SOD* (*cytoSOD*) in 7-day old wild-type (WT) and *latd* mutant roots, with and without 10  $\mu$ M ABA for 24 hours.

**Supplemental Figure S6.** Gene expression analysis for other ROS-related antioxidant genes. *Alternative Oxidase (AOX)*, *Cytosolic Ascorbate Peroxidase (cAPX)*, and *Glutathione S-Transferase (GST)* in 7 day-old wild-type (WT) and *latd* mutant roots with and without 10  $\mu$ M ABA for 24 hours.

**Supplemental Table S1.** Primers used in qRT-PCR.

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## AUTHOR CONTRIBUTIONS



J.M.H. and C.Z. designed the research. C.Z. performed the research and analyzed the data. A. B. contributed tools for analyzing NADPH oxidase expression. C.Z. and J.M.H. wrote the paper.

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## FIGURE LEGENDS

**Figure 1.** *latd* mutant roots have altered ROS levels and localization.

**(A-F)** Representative images of 5 day-old wild-type (WT) and *latd* mutant roots stained with nitroblue tetrazolium (NBT), to indicate superoxide ( $O_2^{\cdot-}$ ). *latd* mutants have increased  $O_2^{\cdot-}$  levels at both the root tips (**A, B**) and the mature region of the roots (**C, D**), taken at 10 mm from root tips, as shown in (**N**).

M, meristem; EZ, elongation zone; DZ, differentiation zone. Scale bars, 1 mm.

**(E-F)** Longitudinal thick sections of NBT-stained mature roots, 5 mm from the tip. Ectopic  $O_2^{\cdot-}$  accumulation in *latd* is observed in the cortical cell end walls (arrowheads) and in the vascular bundle (vb) at this location in the root. Scale bars, 100  $\mu$ m. Sections are 90  $\mu$ m thick.

**(G)** Quantification of NBT staining in wild-type and *latd* mutant roots. The amount of  $O_2^{\cdot-}$  represents nmoles of reduced NBT per gram tissue. Data was collected from three biological replicates with 28 roots total for each genotype. A one-way ANOVA was performed giving a p-value < 0.01.

**(H-K)** Whole-mount 3,3'-diaminobenzidine (DAB) staining of  $H_2O_2$  accumulation in 5 day-old WT and *latd* roots. Root tip staining is similar in both WT and *latd* roots, with staining observed in the meristem. DAB staining is higher throughout the *latd* root, increasing strongly at the end of the DZ (white arrowhead in I). Scale bars, 1 mm.

**(L-M)** Mature root, 10 mm from the root tip (as shown in N) of 7 day-old roots stained with dihydrofluorescein diacetate ( $H_2DCFDA$ ) to label  $H_2O_2$ . Note the occasional brightly labeled epidermal cells in *latd*. Scale bars, 100  $\mu$ m.

All staining above was from at least three biological replicates for a total of 10 to 30 plants total for each condition.

**(N)** Diagram showing the locations of images in this figure. The red bracket indicates the position of root tips (3 mm, containing about 1 mm meristem) and the red arrow indicates the position of the mature region photos (10mm from the tips).

**Figure 2.** *latd* mutant roots are defective in cell elongation.



**(A-B, E-F)** Representative confocal images of propidium iodide-stained root epidermal cells from 5 day-old (A and B) and 21 day-old (E and F) wild-type (WT) (A and E) and *latd* mutant roots (B and F). All images were taken 10 mm from the root tip. Scale bars, 50  $\mu$ m.

**(C-D, G-H)** Average epidermal cell length (C and G) and width (D and H) from 5 day-old (C-D) and 21 day-old (G-H) wild-type and *latd* mutant roots. Graphs display the mean  $\pm$  standard error of  $n > 50$  cells (10 cells per root from at least 5 roots in each genotype) for length measurements,  $n > 100$  cells (20 cells per root from at least 5 roots in each genotype) for width measurements. All measurements were taken at 10 mm distance from the root tip. Asterisks indicate a statistically significant difference with  $p$ -value  $< 0.001$ , by Student's  $t$ -test.

**Figure 3.** ABA lowers superoxide levels in wild-type (WT) and *latd* mutant roots.

**(A)** Whole-mount NBT staining of 5-day old WT A17 and *latd* mutant roots grown continuously with and without 10  $\mu$ M ABA. All NBT staining was repeated four times with at least 30 roots for each condition. Scale bars, 1 mm.

**(B-I)** NBT staining of A17 (B, D, F, H) and *latd* mutant (C, E, G, I) root meristems grown with (D, E, H, I) and without (B, C, F, G) 10  $\mu$ M ABA. B-E, staining of 10 mm distance from the root tips; F-I, root tips (1.5 mm). Scale bars, 1 mm.

**(J)** NBT staining of 21 day-old *latd* mutant roots grown continuously on 0, 1, or 10  $\mu$ M ABA. Note the elongating lateral roots on *latd* plants grown on 10  $\mu$ M ABA, a concentration that restores lateral root growth in the *latd* mutant. Scale bars, 1 mm.

**(K)** Quantification of NBT staining in wild-type (WT) and *latd* roots. Plants were grown on BNM medium with and without 10  $\mu$ M ABA for 7 days. Amount of  $O_2^{\cdot -}$  represents nmoles of reduced NBT per gram tissue. Data was collected from two biological replicates for a total of  $n = 19$  to 33 roots per treatment. Error bars represent standard error. Asterisks represent statistical significance between treatments ( $p$ -value  $< 0.05$ ) using one-way ANOVA.

**Figure 4.** Epidermal cell length (A) and width (B) of *latd* mutant roots can be increased by adding 10  $\mu$ M ABA. For each genotype in each treatment, graphs display the mean of  $n = 50$  to 60 cells from at least 5 roots for cell length;  $n = 102$  to 126 cells from at least 5 roots for cell width. Error bars represent standard error and letters indicate a statistically significant difference between different treatments and genotypes, with  $p$ -value  $< 0.05$ , using Student's  $t$ -test.

**Figure 5.** Decreasing ROS levels in *latd* mutants rescues both cell and lateral root elongation defects.

**(A)** Epidermal cell length in WT and *latd* mutant roots, treated with 0.1  $\mu$ M diphenylene iodonium (DPI) for 2 days. Plants were first grown in plain BNM medium for 8 days in growth pouches, then BNM supplemented with 0.1  $\mu$ M DPI was added into the pouches. Graphs display the mean of n=40 to 80 cells from at least 4 roots for each genotype. Cell length was measured in cells 1 cm from the root tip. Error bars represent standard error. Letters indicate a statistically significant difference between genotypes and treatments with p-value <0.05, using Student's t-test.

**(B)** NBT staining in WT and *latd* roots grown on BNM medium +/- 0.1  $\mu$ M DPI for 2 days. Note decreased  $O_2^{\cdot-}$  staining in DPI-treated roots. Experiment was repeated twice with at least 5 roots for each condition. Scale bars, 1 mm.

**(C)** 21 day-old *latd* mutant plants in growth pouches grown continuously with either control medium (left panel) or medium containing 1  $\mu$ M DPI (right panel). Scale bars, 1 cm. Note increased lateral root length on DPI-treated roots.

**(D)** The percentage of WT and *latd* mutant plants grown as in C that develop lateral roots longer than 2 mm. Plants were grown for 21 days with either control medium or medium supplemented with 1  $\mu$ M DPI, or 2  $\mu$ M EUK 134 (SODm). Data is from 2 biological replicates with n > 10 for each column.

**Figure 6.** *latd* mutant roots have altered expression of several ROS-related genes.

**(A)** Relative expression of *MtRbohC* in WT and *latd* mutant roots.

**(B)** Relative expression of six other *MtRboh* genes in WT and *latd* mutant roots.

**(C)** Relative expression of a putative apoplastic *Cu/ZnSOD* (*ApoCu/ZnSOD*) in WT and *latd* mutant roots.

**(D)** Relative expression of a putative cell wall localized peroxidase, *cwPRX2* in WT and *latd* mutant roots.

**(E)** Relative expression of *RIP1* in WT and *latd* mutant roots.

Whole root tissue of 7 day-old plants grown continuously on BNM +/-10  $\mu$ M ABA was harvested and analyzed by qRT-PCR. Graphs represent the mean +/- standard error from three biological replicates, with n=20 for each genotype in each treatment per replicate. One-way ANOVA was

performed to analyze statistical differences (p-value <0.05) for each gene examined. Different letters indicate statistically significant difference between genotypes and treatments.

Primers used for qRT-PCR are listed in Supplemental Table 1.

**Figure 7.** Silencing *MtRbohC* expression via RNAi increases root elongation and lateral root density.

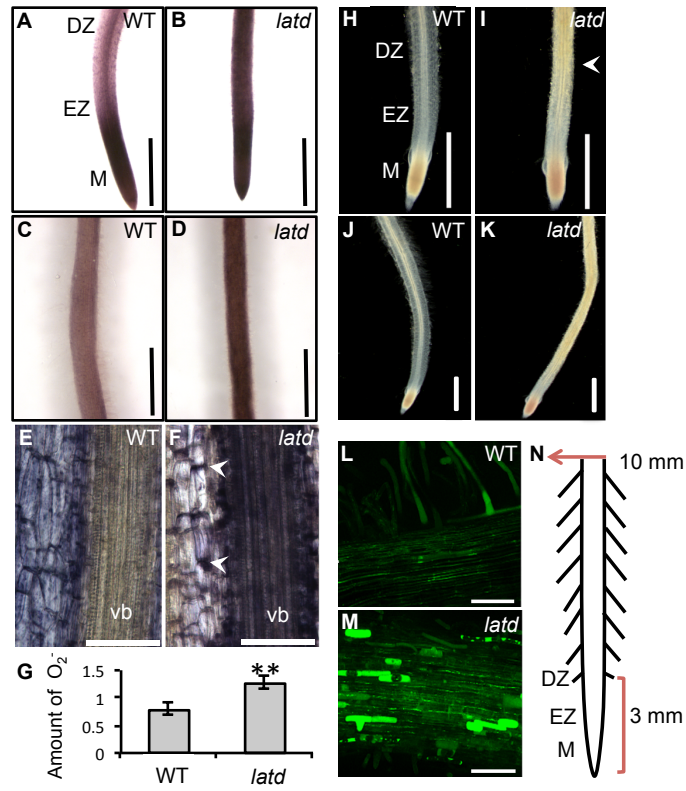
**(A-D)** Representative images of wild-type (WT) (A, B) and *latd* mutants (C, D) transformed with control vector (GUS RNAi) (A, C) and *MtRbohC* RNAi vector (B, D) using *Agrobacterium rhizogenes* transformation. Scale bars, 1 cm.

**(E-F)** Primary root length (cm) (E) and lateral root density (LRD) (F) in wild-type (WT) and *latd* mutant roots transformed with the *MtRbohC* RNAi vector. LRD, lateral root number per cm primary root. Graph represents the average of four biological replicates, with n=53 to 80 individual transformants total for each genotype and treatment. Error bars represent standard error, asterisks indicate a statistically significant difference using one-way ANOVA, p-value < 0.05.

**(G)** Percentage of *latd* transformants that developed lateral root (LR) longer than 2mm. Graph represents data from four biological replicates (Experiment #1-4). For *latd* plants transformed with GUS RNAi, n=6, 23, 13 and 11 for Experiment 1-4 respectively; for *latd* plants transformed with *MtRbohC* RNAi, n=7, 18, 16 and 12 respectively.

**(H)** Relative expression of *MtRbohC*, *MtRbohD*, *MtRbohB* and *MtRbohA* in wild-type (WT) plants transformed with the control vector and the *MtRbohC* RNAi vector. Graph shows relative expression from 2 individual transformants for each vector. Note that only *MtRbohC* expression is knocked down in WT roots by the *MtRbohC* RNAi vector, and not the closely related *MtRbohD* gene, nor *MtRbohA* or *MtRbohB*, indicating the specificity of this approach. Error bars represent standard error from two technical replicates.

## FIGURES



**Figure 1. *latd* mutant roots have altered ROS levels and localization.**

(A-F) Representative images of 5 day-old wild-type (WT) and *latd* mutant roots stained with nitroblue tetrazolium (NBT), to indicate superoxide ( $O_2^{\cdot -}$ ). *latd* mutants have increased  $O_2^{\cdot -}$  levels at both the root tips (A, B) and the mature region of the roots (C, D), taken at 10 mm from root tips, as shown in (N).

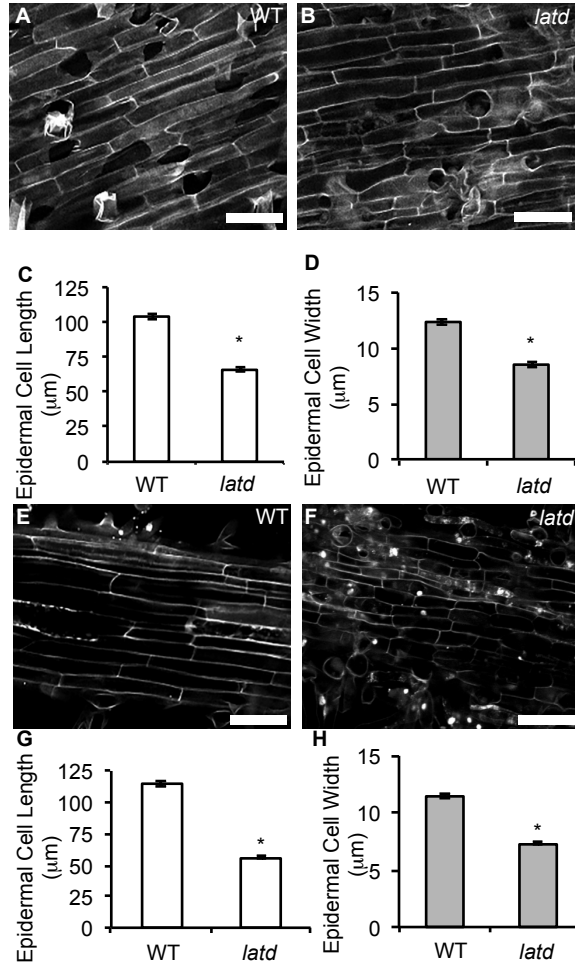
M, meristem; EZ, elongation zone; DZ, differentiation zone. Scale bars, 1 mm.

(E-F) Longitudinal thick sections of NBT-stained mature roots, 5 mm from the tip. Ectopic  $O_2^{\cdot -}$  accumulation in *latd* is observed in the cortical cell end walls (arrowheads) and in the vascular bundle (vb) at this location in the root. Scale bars, 100  $\mu$ m. Sections are 90  $\mu$ m thick.

**(G)** Quantification of NBT staining in wild-type and *latd* mutant roots. The amount of  $O_2^{\cdot -}$  represents nmoles of reduced NBT per gram tissue. Data was collected from three biological replicates with 28 roots total for each genotype. A one-way ANOVA was performed giving a p-value < 0.01.

**(H-K)** Whole-mount 3,3'-diaminobenzidine (DAB) staining of  $H_2O_2$  accumulation in 5 day-old WT and *latd* roots. Root tip staining is similar in both WT and *latd* roots, with staining observed in the meristem. DAB staining is higher throughout the *latd* root, increasing strongly at the end of the DZ (white arrowhead in I). Scale bars, 1 mm.

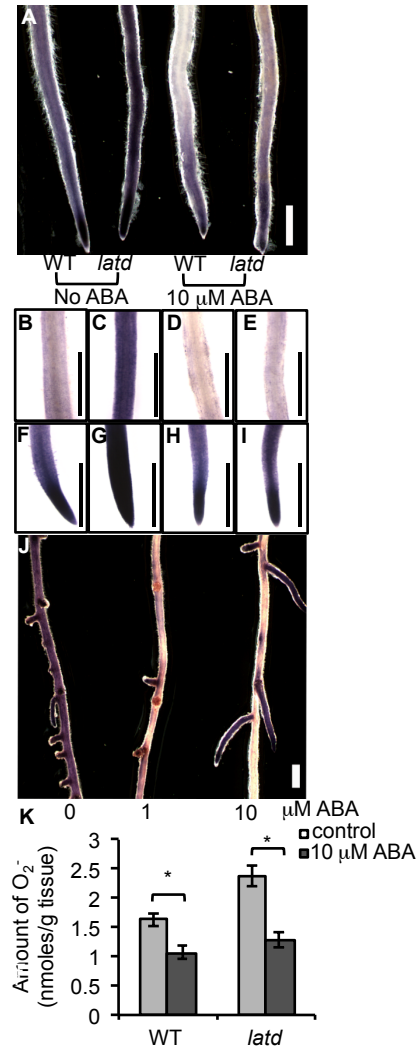
**(L-M)** Mature root, 10 mm from the root tip (as shown in N) of 7 day-old roots stained with dihydrofluorescein diacetate ( $H_2DCFDA$ ) to label  $H_2O_2$ . Note the occasional brightly labeled epidermal cells in *latd*. Scale bars, 100  $\mu m$ .



**Figure 2. *latd* mutant roots are defective in cell elongation.**

(A-B, E-F) Representative confocal images of propidium iodide-stained root epidermal cells from 5 day-old (A and B) and 21 day-old (E and F) wild-type (WT) (A and E) and *latd* mutant roots (B and F). All images were taken 10 mm from the root tip. Scale bars, 50 μm.

(C-D, G-H) Average epidermal cell length (C and G) and width (D and H) from 5 day-old (C-D) and 21 day-old (G-H) wild-type and *latd* mutant roots. Graphs display the mean +/- standard error of n>50 cells (10 cells per root from at least 5 roots in each genotype) for length measurements, n>100 cells (20 cells per root from at least 5 roots in each genotype) for width measurements. All measurements were taken at 10 mm distance from the root tip. Asterisks indicate a statistically significant difference with p-value <0.001, by Student's t-test.



**Figure 3. ABA lowers superoxide levels in wild-type and *latd* mutant roots.**

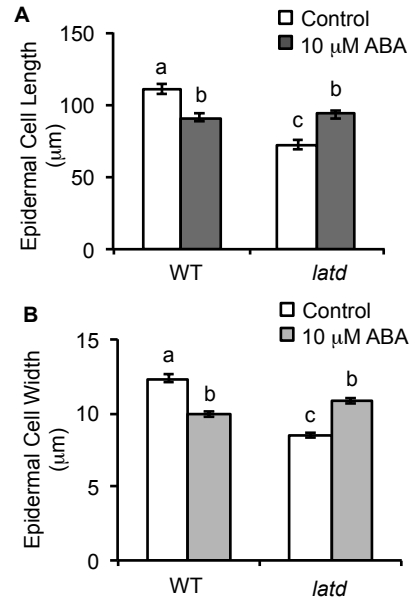
**(A)** Whole-mount NBT staining of 5-day old wild-type (WT) A17 and *latd* mutant roots grown continuously with and without 10 μM ABA. All NBT staining was repeated four times with at least 30 roots for each condition. Scale bars, 1 mm.

**(B-I)** NBT staining of A17 (B, D, F, H) and *latd* mutant (C, E, G, I) root meristems grown with (D, E, H, I) and without (B, C, F, G) 10 μM ABA. B-E, staining of 10 mm distance from the root tips; F-I, root tips (1.5 mm). Scale bars, 1 mm.

**(J)** NBT staining of 21 day-old *latd* mutant roots grown continuously on 0, 1, or 10  $\mu\text{M}$  ABA. Note the elongating lateral roots on *latd* plants grown on 10  $\mu\text{M}$  ABA, a concentration that restores lateral root growth in the *latd* mutant. Scale bars, 1 mm.

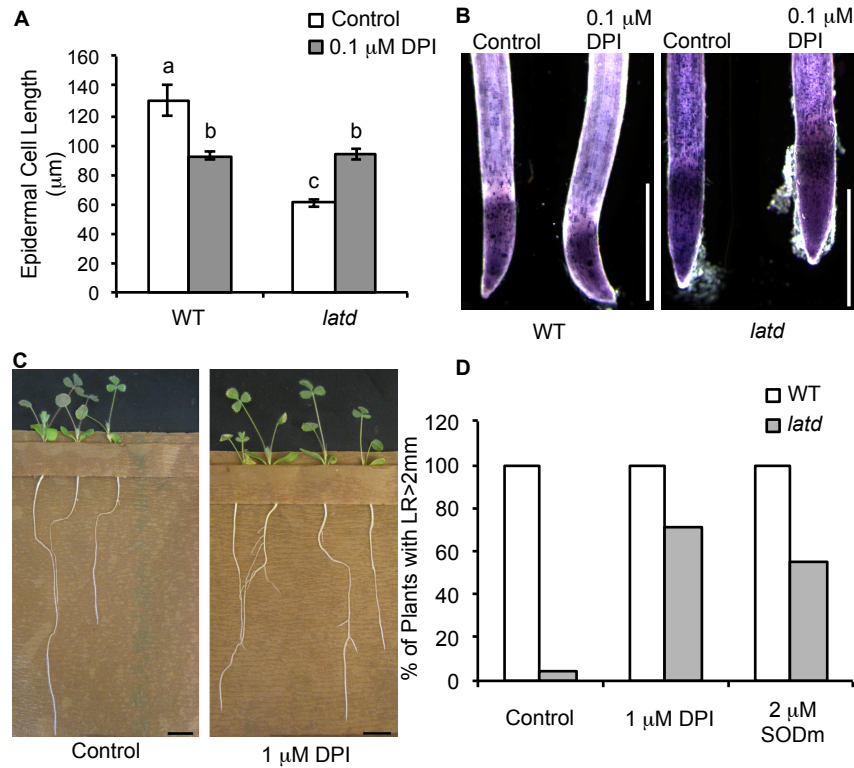
**(K)** Quantification of NBT staining in wild-type (WT) and *latd* roots. Plants were grown on BNM medium with and without 10  $\mu\text{M}$  ABA for 7 days. Amount of  $\text{O}_2^{\cdot -}$  represents nmoles of reduced NBT per gram tissue. Data was collected from two biological replicates for a total of n= 19 to 33 roots per treatment. Error bars represent standard error. Asterisks represent statistical significance between treatments (p-value < 0.05) using one-way ANOVA.





**Figure 4. Epidermal cell elongation and width of *latd* mutant roots can be rescued by ABA.**

Epidermal cell length (A) and width (B) of *latd* mutant roots can be increased by adding 10  $\mu$ M ABA. For each genotype in each treatment, graphs display the mean of n=50 to 60 cells from at least 5 roots for cell length; n=102 to 126 cells from at least 5 roots for cell width. Error bars represent standard error and letters indicate a statistically significant difference between different treatments and genotypes, with p-value <0.05, using Student's t-test.



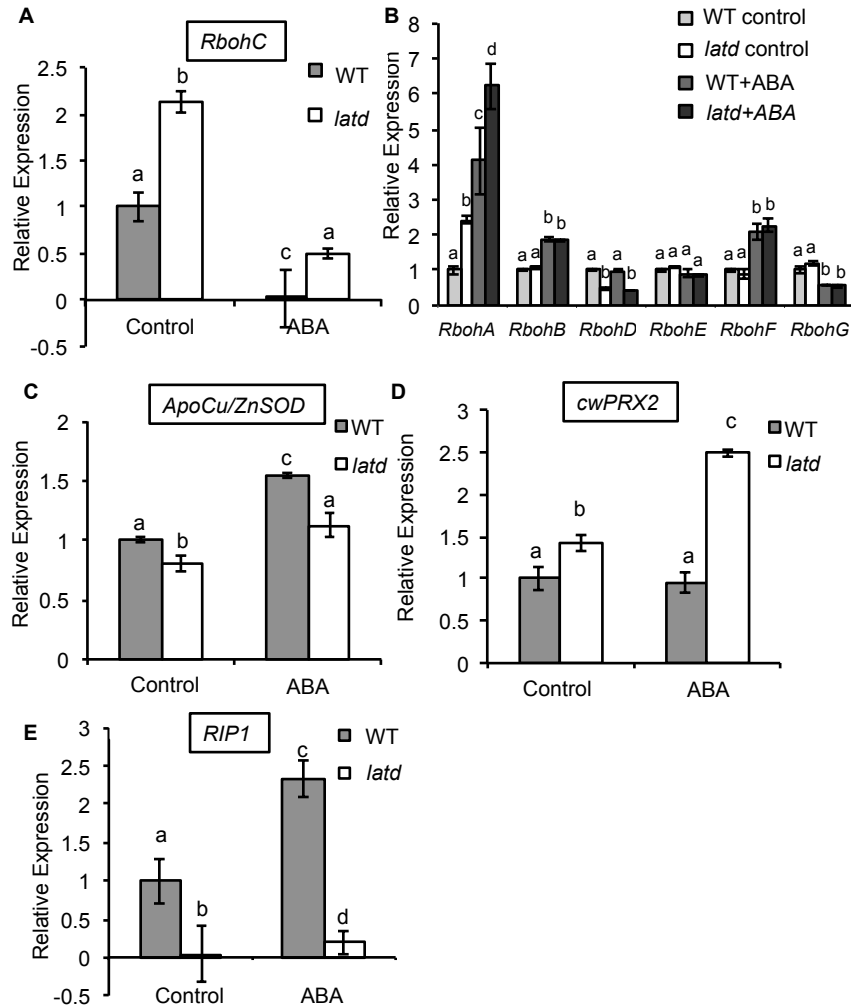
**Figure 5. Decreasing ROS levels in *latd* mutants rescues both cell and lateral root defects.**

**(A)** Epidermal cell length in WT and *latd* mutant roots, treated with 0.1 μM diphenylene iodonium (DPI) for 2 days. Plants were first grown in plain BNM medium for 8 days in growth pouches, then BNM supplemented with 0.1 μM DPI was added into the pouches. Graphs display the mean of n=40 to 80 cells from at least 4 roots for each genotype. Cell length was measured in cells 1 cm from the root tip. Error bars represent standard error. Letters indicate a statistically significant difference between genotypes and treatments with p-value <0.05, using Student's t-test.

**(B)** NBT staining in WT and *latd* roots grown on BNM medium +/- 0.1 μM DPI for 2 days. Note decreased O<sub>2</sub><sup>-</sup> staining in DPI-treated roots. Experiment was repeated twice with at least 5 roots for each condition. Scale bars, 1 mm.

**(C)** 21 day-old *latd* mutant plants in growth pouches grown continuously with either control medium (left panel) or medium containing 1  $\mu$ M DPI (right panel). Scale bars, 1 cm. Note increased lateral root length on DPI-treated roots.

**(D)** The percentage of WT and *latd* mutant plants grown as in C that develop lateral roots longer than 2 mm. Plants were grown for 21 days with either control medium or medium supplemented with 1  $\mu$ M DPI, or 2  $\mu$ M EUK 134 (SODm). Data is from 2 biological replicates with  $n > 10$  for each column.



**Figure 6. *latd* mutant roots have altered expression of several ROS-related genes.**

**(A)** Relative expression of *MtrbohC* in WT and *latd* mutant roots.

**(B)** Relative expression of six other *Mtrboh* genes in WT and *latd* mutant roots.

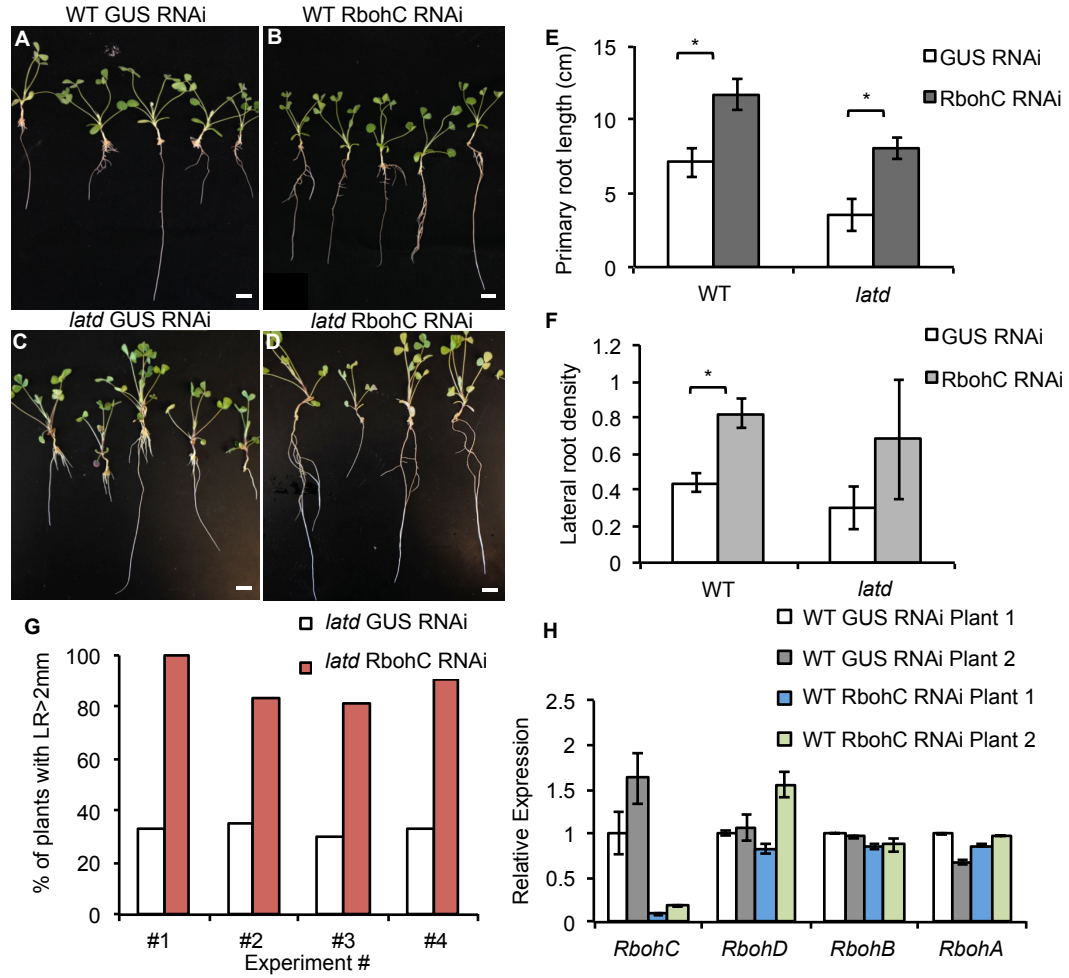
**(C)** Relative expression of a putative apoplasmic *Cu/ZnSOD* (*ApoCu/ZnSOD*) in WT and *latd* mutant roots.

**(D)** Relative expression of a putative cell wall localized peroxidase, *cwPRX2* in WT and *latd* mutant roots.

**(E)** Relative expression of *RIP1* in WT and *latd* mutant roots.

Whole root tissue of 7 day-old plants grown continuously on BNM +/-10  $\mu$ M ABA was harvested and analyzed by qRT-PCR. Graphs represent the mean +/- standard error from three biological replicates, with n=20 for each genotype in each treatment per replicate. One-way ANOVA was performed to analyze statistical differences (p-value <0.05) for each gene examined. Different letters indicate statistically significant difference between genotypes and treatments.

Primers used for qRT-PCR are listed in Supplemental Table 1.



**Figure 7. Silencing *MtRbohC* expression via RNAi increases root elongation and lateral root density.**

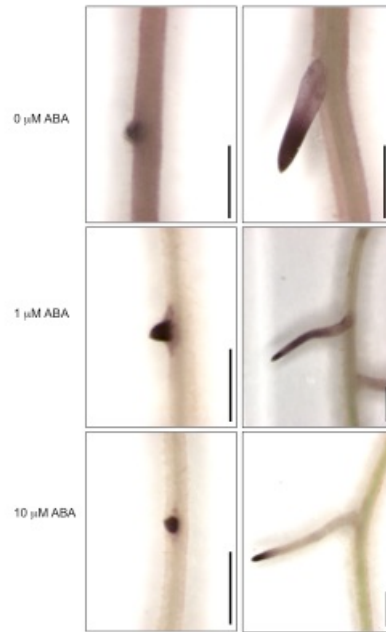
(A-D) Representative images of wild-type (WT) (A, B) and *latd* mutants (C, D) transformed with control vector (GUS RNAi) (A, C) and *MtRbohC* RNAi vector (B, D) using *Agrobacterium rhizogenes* transformation. Scale bars, 1 cm.

(E-F) Primary root length (cm) (E) and lateral root density (LRD) (F) in wild-type (WT) and *latd* mutant roots transformed with the *MtRbohC* RNAi vector. LRD, lateral root number per cm primary root. Graph represents the average of four biological replicates, with n=53 to 80 individual transformants total for each genotype and treatment. Error bars represent standard

error, asterisks indicate a statistically significant difference using one-way ANOVA, p-value < 0.05.

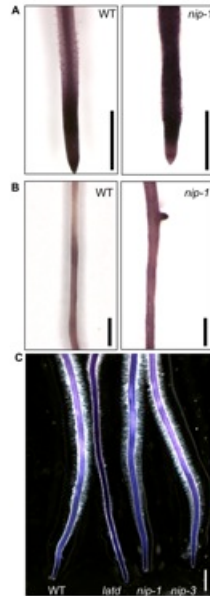
**(G)** Percentage of *latd* transformants that developed lateral root (LR) longer than 2mm. Graph represents data from four biological replicates (Experiment #1-4). For *latd* plants transformed with GUS RNAi, n=6, 23, 13 and 11 for Experiment 1-4 respectively; for *latd* plants transformed with MtRbohC RNAi, n=7, 18, 16 and 12 respectively.

**(H)** Relative expression of *MtRbohC*, *MtRbohD*, *MtRbohB* and *MtRbohA* in wild-type (WT) plants transformed with the control vector and the MtRbohC RNAi vector. Graph shows relative expression from 2 individual transformants for each vector. Note that only *MtRbohC* expression is knocked down in WT roots by the MtRbohC RNAi vector, and not the closely related *MtRbohD* gene, nor *MtRbohA* or *MtRbohB*, indicating the specificity of this approach. Error bars represent standard error from two technical replicates.

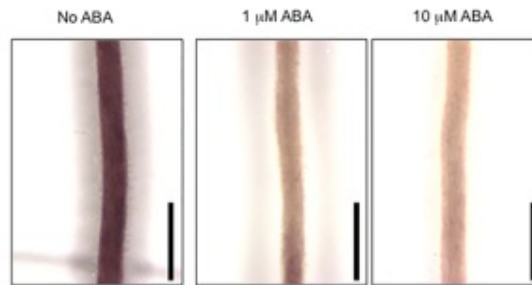


**Supplemental Figure S1.** Seven-day old wild-type lateral roots at different developmental stages stained for  $O_2^{\bullet -}$  with NBT. Scale bars, 1 mm.

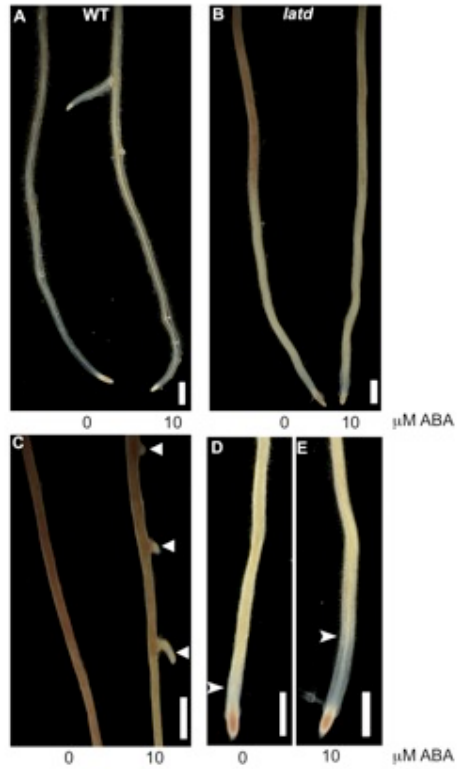




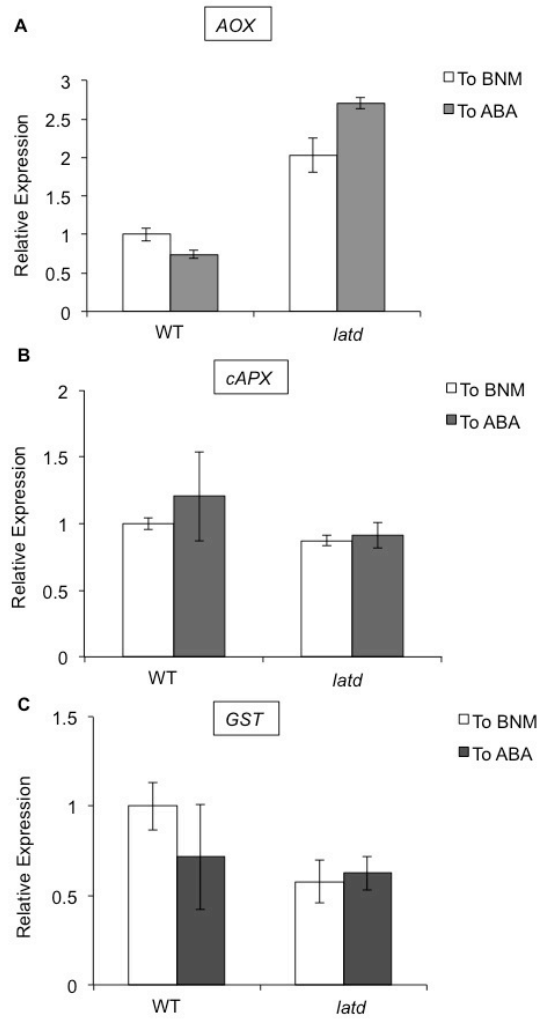
**Supplemental Figure S2.** The *nip-1* allele of the *LATD/NIP* gene has increased superoxide ( $O_2^{\cdot-}$ ) levels. NBT staining of  $O_2^{\cdot-}$  in 7-day old *nip-1* mutant root tips (A) and mature roots (10 mm from root tip) (B). Graded ROS staining in *latd*, *nip-1* and *nip-3* mutant plants reveals an allelic series of excessive ROS production that corresponds to the severity of the root growth defect (Yendrek et al., 2010) (C).



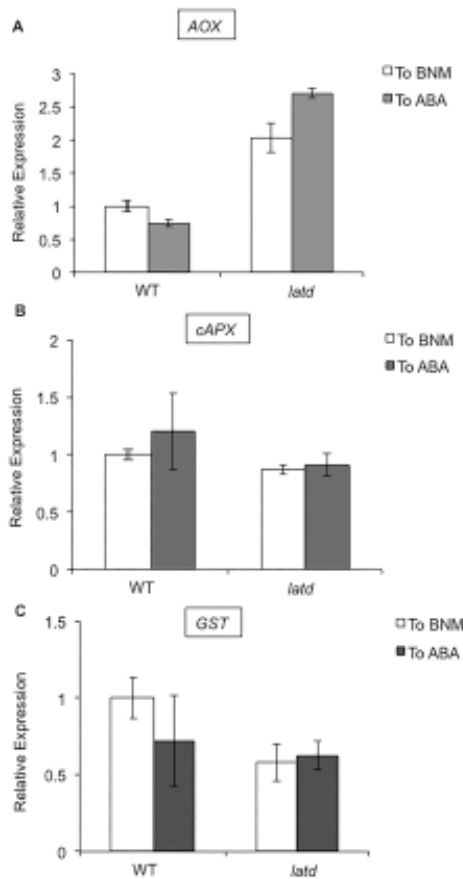
**Supplemental Figure S3.** ABA decreases superoxide levels in *nip-1* roots, as it does for *latd* roots. Seven-day old plants grown on control BNM medium were transferred to different concentrations of ABA, as indicated, for 48 hours, then stained with NBT. ABA decreases  $O_2^{\cdot -}$  levels in *nip-1* mutant mature roots in the mature zone (10 mm from root tip).



**Supplemental Figure S4.** DAB staining of H<sub>2</sub>O<sub>2</sub> in 5-day old wild-type (A) and *latd* (B) roots grown with and without 10 μM ABA. ABA can decrease H<sub>2</sub>O<sub>2</sub> levels in *latd* roots, mostly in the mature region of the root (B). Note 10 μM ABA decreases H<sub>2</sub>O<sub>2</sub> in the region where rescued lateral root growth is observed in *latd* mutants (C). The lower boundary of DAB staining in the mature region of *latd* roots shifts shootward after growth on 10 μM ABA, indicated by arrowheads in (D, E).



**Supplemental Figure S5.** Relative Expression of *FeSOD* (A) and a cytoplasmic *SOD* (*cytoSOD*) (B) in 7-day old wild-type (WT) and *latd* mutant roots, with and without 10  $\mu$ M ABA for 24 hours. Whole root tissue was harvested and the graphs represent the mean  $\pm$  standard error from three biological replicates, with  $n=20$  for each genotype per treatment in each replicate. There is no significant difference between genotypes or treatments, using one-way ANOVA.



**Supplemental Figure S6. Gene expression analysis for other ROS-related antioxidant genes.**

Relative expression of *Alternative Oxidase* (AOX) (A), *Cytosolic Ascorbate Peroxidase* (cAPX) (B), and *Glutathione S-Transferase* (GST) (C) in the specified treatments as compared to wild-type. Seven day-old wild-type (WT) and *latd* mutant plants were transferred either to control BNM medium or to BNM supplemented with 10  $\mu$ M ABA. Whole root tissue was harvested 24 hours post transfer. Data represents an average from three biological replicates, with n=20 for each genotype per treatment in each replicate. There is no statistically significant difference relative to WT using one-way ANOVA, p-value <0.05.

Supplemental Table S1. Primers used in qRT-PCR.

Gene	Accession No. in Mt4.0	Forward (5'-3')	Reverse (5'-3')	Reference
<i>RbohA</i>	Medtr1g083290	GCGCAACTCCTT TGATTAGC	GAAATAGGCTCG CTTGGTTG	(Marino et al., 2011)
<i>RbohB</i>	Medtr3g098380	GCTCGCTCTGCT CTTATTGC	TGCGCTTGTAGA CACTACGC	
<i>RbohC</i>	Medtr3g098350	GGGAGACCTGAT GCTATTCAAG	TGTCTTCAACAA TAATGTCCATCT G	
<i>RbohD</i>	Medtr3g098320	ACATGGCTCAGG AGCAAGAC	TGAAGAAGGCGT GGAAAGTC	
<i>RbohE</i>	Medtr8g095520	CCAAATTCTCAC AGGCTTGC	CTCCATACGGAC CATCAATC	
<i>RbohF</i>	Medtr7g060540	GAAGAGCCTTTG TCAAGAGTTTAG	GGATTGATGCTT TAGCTGTGTG	
<i>RbohG</i>	Medtr7g113130	GTGATGGATGAA ATCGCAGA	TGGCATGATGAA GTGATTGA	
<i>ApoCu/ZnSOD</i>	Medtr6g029200	GGGCCTGCAAGA ATGTTACC	CCTGAGGATGAG ACTCGACA	Designed in this study
<i>CytoCu/ZnSOD</i>	Medtr7g114240	CCTGAGGATGAG ACTCGACA	GAACAACAACA GCCCTTCCT	
<i>cwPRX2</i>	Medtr2g084000	GGAAGAAGGGA TAGTCTAGAGGC A	GGAGCTGGGACC TGGAAGAT	
<i>cAPX</i>	Medtr4g061140	GGTCGCTTGCCT GATGC	CCACCCAACAAC TCCGTAAA	(Filippou et al., 2011)
<i>GST</i>	Medtr1g026140	TGGCAAAGGCTT CAATAGAT	AGCAGCAGCCAC CAAGTAGT	
<i>AOX</i>	Medtr5g026620	TTGGATCGAAGA TGATGATGAG	ACCAGCACCACC CTGAGAC	
<i>FeSOD</i>	Medtr1g048990	TCTTGCAACTGA GGAGGA	AGGACGCCGATT CTGATA	
<i>RIP1</i>	Medtr5g074860	TAGGGAAAAAC GCATTGGAG	ACAACAGGGCCT TTGCATAC	(Ding et al., 2008)
<i>UBC9</i>	Medtr7g116940	GGTTGATTGCTC TTCTCTCCCC	AAGTGATTGCTC GTCCAACCC	(Kakar et al., 2008)
<i>PDF2</i>	Medtr6g084690	GTGTTTTGCTTC CGCCGTT	CCAAATCTTGCT CCCTCATCTG	

**CHAPTER THREE: IDENTIFICATION OF DOWNSTREAM REGULATORS IN ABA  
SIGNALING MEDIATED BY LATD/NIP IN MEDICATO TRUNCATULA VIA  
TRANSCRIPTION FACTOR PROFILING**

## ABSTRACT

The plant hormone abscisic acid (ABA) plays an important role in regulating root development, and coordinates environmental signals with plant genetic networks to modulate root architecture. In the model legume *Medicago truncatula*, ABA inhibits primary root elongation but stimulates lateral root formation. The nitrate transporter MtLATD/NIP is required for root and nodule development; disruption in this gene leads to severe growth defects that can be rescued by ABA application. However, the downstream effectors of ABA signaling that restore the *latd* root defects are not known. ABA regulates gene expression via transcription factors (TFs). To identify the TFs downstream of ABA and the MtLATD/NIP signaling pathway, we used a high-throughput qRT-PCR based TF profiling against 1,084 TF targets. We found 20 TFs that exhibit altered expression levels in *latd* mutants, 60% of which can be restored to wild-type levels by ABA. Curiously, TF targets whose expression is regulated by ABA are very different in *latd* and wild-type roots, suggesting that MtLATD/NIP is crucial for mediating ABA regulation of TF expression. Moreover, ABA changes the TFs regulated by MtLATD/NIP almost completely, indicating a tight control of ABA on MtLATD/NIP-regulated TFs. Surprisingly, we found that the expression of *NODULATION SIGNALING PATHWAY 2* (MtNSP2), a GRAS family TF required for nodulation, is regulated by MtLATD/NIP, ABA and nitrate. We show that MtNSP2 regulates the expression a subset of genes involved in ABA metabolism and signaling, suggesting that MtNSP2 may have a novel function in controlling ABA homeostasis, or mediating ABA signaling and responses. Lastly, we found that induction of MtNSP2 and its downstream targets by cytokinin or by *Sinorhizobium meliloti* both require MtLATD/NIP. This finding indicates MtLATD/NIP functions in early nodulation signal transduction by regulating MtNSP2, and mediates crosstalk with cytokinin.



## INTRODUCTION

Plants live in an environment that constantly presents the challenges of nutrient availability and abiotic stresses such as drought and high salinity. Plant root systems develop to maximize their ability to reach the limited water and nutrients in the soil, and adjust patterns of root growth quickly, in response to environmental conditions for survival (Petricka et al., 2012). The plasticity of plant root architecture is a combination of both genetic regulation and integration of environmental cues (Malamy, 2005). The plant hormone abscisic acid (ABA) plays an important role in mediating root development in response to various abiotic stresses (Finkelstein, 2013). Under stress conditions such as drought, ABA synthesis is stimulated, and ABA levels rapidly increase. Then ABA synthesized in roots can be transported through the vasculature into the shoot to promote stomatal closure, thereby preventing further water loss (Finkelstein, 2013). ABA also accumulates during seed maturation and in this way controls seed dormancy (Nakashima and Yamaguchi-Shinozaki, 2013). However, the exact role of ABA in root development is still not completely clear, and it seems to have opposite effects on root growth under different environmental conditions and in different plant species. In *Arabidopsis thaliana*, exogenous ABA inhibits primary root growth and lateral root development (Zeevaart and Creelman, 1988; De Smet et al., 2003). However, the root growth defects in some of the ABA-deficient mutants also reveal a positive role for endogenous ABA in maintaining and promoting root growth (Cheng et al., 2002; Barrero et al., 2005). In fact, applying ABA exogenously can rescue the root growth phenotypes of both *Arabidopsis aba2* mutants and *Medicago truncatula latd* mutants (Cheng et al., 2002; Liang et al., 2007). Low concentrations of ABA (in the nanomolar range) have been shown to preserve the meristem and promote root growth by promoting the quiescence of the quiescent center (QC) in the primary root apical meristem (RAM) and suppressing stem cell differentiation (Zhang et al., 2010). In addition, at low water

potentials, ABA is required for maintaining root growth in maize seedlings (Sharp et al., 1994; Sharp et al., 2004). Interestingly, in certain taxa ABA can either inhibit or induce lateral root formation, reflecting a change in the ABA response acquired at the base of the legume lineage (Liang and Harris, 2005).

ABA is perceived by ABA receptors of the PYR/PYL/RCAR family, which then form a complex with Protein Phosphatase 2C (PP2C), thus releasing the inhibition of PP2C on ABA signaling (Ma et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Park et al., 2009). The activated subclass III SNF1-related protein kinase 2 (SnRK2) transduce the signal by phosphorylating a variety of substrates, including transcription factors (TFs) (Yoshida et al., 2002; Yoshida et al., 2006; Fujita et al., 2009; Nakashima et al., 2009; Fujita et al., 2013). TFs that function in ABA signaling have been identified and studied extensively in *Arabidopsis*, and many members from different TF families have been shown to play roles in regulating ABA-responsive gene expression and ABA-mediated stress tolerance (Golldack et al., 2014). In particular, the bZIP transcription factors, ABRE-Binding Protein/ABRE-Binding Factors (AREB/ABF), are major regulators in ABA-mediated osmotic stress responses (Uno et al., 2000). AREB/ABF TFs recognize and bind to the *cis*-acting ABA-responsive element (ABRE), which is found in the promoters of many ABA-responsive genes, then regulate transcription (Yoshida et al., 2010; Yoshida et al., 2014). Members of the DREB/DBF subfamily of the AP2/ERF TFs recognize the drought-responsive element (DRE), which is also present in the promoter of many stress-related genes (Yamaguchi-Shinozaki and Shinozaki, 1994; Yamaguchi-Shinozaki and Shinozaki, 2006). Besides these two TF families, members of many other TF families such as NAC, bHLH, MYB, WRKY, HD-ZIP, are also involved in ABA and stress responses (Golldack et al., 2014).

Legume species have evolved a special ability to establish a mutual symbiotic relationship with nitrogen-fixing bacteria in the soil and form a specialized root organ, the nodule,

where nitrogen fixation takes place. This symbiotic relationship is crucial for plant growth because it provides nitrogen in a form that can be assimilated by the host plant (Oldroyd et al., 2011). The initial signal exchange is mediated by legume root-secreted flavonoids and rhizobially produced lipochitooligosaccharides (LCOs), called Nodulation (Nod) factor (Oldroyd, 2013). A successful signal exchange will ultimately lead to root hair deformation and rhizobia entrapment, the formation of an infection structure, the infection thread, with the simultaneous occurrence of cell division in the cortex, which results in the formation of the nodule primordium (Oldroyd and Downie, 2008). Nod factor perception triggers calcium spiking in responsive root cells, which induces changes in gene expression (Ehrhardt et al., 1996; Gleason et al., 2006). Downstream of calcium spiking there are two pathways, one in the epidermis, that appears to mediate infection, and one in the cortex, that leads to cell division and the formation of a nodule primordium (Oldroyd et al., 2011). Several TFs have been shown to be essential for nodulation in both bacterial infection in the epidermis and nodule organogenesis in the cortex: two GRAS family TFs, *M. truncatula* NODULATION SIGNALING PATHWAY 2 (NSP2) and NSP1, form a heterocomplex that binds to the promoters of Nod factor-inducible genes such as *EARLY NODULIN 11* (*ENOD11*) in the epidermis (Hirsch et al., 2009). The MtNSP2 and MtNSP1 complex also induces the expression of TFs *ERF REQUIRED FOR NODULATION 1* (*ERN1*) and *NODULE INCEPTION* (*NIN*), which are both required for bacterial infection and nodule formation (Marsh et al., 2007; Middleton et al., 2007; Cerri et al., 2012).

The plant hormone cytokinin is a major regulator that induces cortical responses in nodule organogenesis (Cooper and Long, 1994; Gonzalez-Rizzo et al., 2006; Murray et al., 2007). Exogenous cytokinin can induce the expression of genes in the cortical pathway, and is associated with cell division, leading to the formation of the nodule primordium (Fang and Hirsch, 1998; Oldroyd and Downie, 2008). Interestingly, activation of cytokinin signaling leads to binding of

RESPONSE REGULATOR 1 (RR1) to the promoter of *MtNSP2* where it induces *MtNSP2* expression, indicating that *MtNSP2* is a direct target of cytokinin signaling (Ariel et al., 2012).

Nodulation is an energy-consuming process and is sensitive to ABA-mediated abiotic stresses, such as drought and high salinity, which can inhibit nodulation (Arrese-Igor et al., 1999; Zahran, 1999; Gil-Quintana et al., 2013). ABA negatively regulates nodulation by inhibiting calcium spiking in growing root hairs (Ding et al., 2008). ABA also acts antagonistically to cytokinin signaling during nodulation, by repressing cytokinin-inducible nodulation gene expression (Ding et al., 2008). Many TFs that function in Nod factor signaling and nodule formation have been identified; however, TFs that mediate ABA and stress responses in *M. truncatula* are still few in number. The expression of TFIIIA-like C<sub>2</sub>H<sub>2</sub> zinc finger TFs, *MtZpt2-1* and *MtZpt2-2*, are highly inducible by salt stress, and overexpressing these TFs leads to increased root length under high salt condition in *M. truncatula* 108-R genotype (De Lorenzo et al., 2007). The expression of *MtCBF4*, a TF from the AP2/EREBP TF family, is rapidly induced by ABA in leaves, as well as cold, salt and drought stress (Li et al., 2011). Overexpressing *MtCBF4* also increased root growth under salt stress (Li et al., 2011). Only one gene, a NAC family TF, *MtNAC969*, not only negatively regulates salt stress responses in roots, but also plays a role in nodule senescence, thus linking abiotic stress and nodulation (De Zélicourt et al., 2012).

*MtLATD/NIP/MtNPF1.7* encodes a nitrate transporter that belongs to the NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER Family (NPF), and is required for root and nodule meristem function (Bright et al., 2005; Bagchi et al., 2012; L  ran et al., 2014). *latd* mutants exhibit severe root growth phenotypes, with an extremely short primary root, and lateral roots that become arrested shortly after emergence (Bright et al., 2005). Nodules in *latd* mutants are white and small, with no nitrogen fixation activity and reduced infection (Bright et al., 2005). The three alleles of *MtLATD/NIP* exhibit a phenotypic gradient for phenotypes such as bacterial infection: the *latd* mutant exhibits the strongest phenotype with limited infection thread

progression and bacterial release, the *nip-3* mutant exhibits much more wild-type-like phenotypes, and *nip-1* is intermediate. The nitrate transport functions of proteins encoded by the three alleles are consistent with the phenotypic gradient, with only *nip-3* having a nitrate transport activity in *Xenopus* oocytes (Bagchi et al., 2012). When expressing the proteins encoded by the different alleles in *Arabidopsis chl1-5* mutants, defective in the nitrate transporter *AtNRT1.1* gene, only the *nip-3* allele can restore the sensitivity to chlorite (Salehin et al., 2013). Exogenous application of ABA can rescue *latd* root elongation defects, restoring the growth of both primary and lateral roots (Liang et al., 2007). We have recently shown that Reactive Oxygen Species (ROS) levels are increased in *latd* roots, which can also be rescued by ABA (Zhang et al., 2014). Moreover, the superoxide-generating enzyme, *Respiratory Burst Oxidase C* (*MtRbohC*), expression is higher in *latd* roots; applying ABA can decrease its expression to wild-type levels (Zhang et al., 2014). Silencing *MtRbohC* expression in *latd* roots using RNAi leads to increased elongation of the primary root and a higher number of elongated lateral roots (Zhang et al., 2014). However, how does ABA rescue the expression of *MtRbohC* in *latd* mutants? What are the transcriptional regulators that function downstream of ABA to rescue *latd* root phenotypes? Does MtLATD/NIP affect gene expression via regulating the expression of particular TFs? What is MtLATD/NIP function in nodule formation? In order to search for possible answers for these questions, we adopted a high-throughput TF profiling approach to look for potential TFs that function downstream of MtLATD/NIP and ABA in *M. truncatula* roots.

High-throughput quantitative reverse transcription-polymerase chain reaction (qRT-PCR) based TF profiling has become a powerful tool to identify potential important TFs functioning in different processes, and has proved to be a more sensitive method than microarrays in detecting low-abundance TF transcripts (Kakar et al., 2008). Using this method, forty-six TF genes have been identified to be salt stress-regulated in *M. truncatula* roots (Gruber et al., 2009). The same approach was also used for isolating TFs involved in desiccation tolerance for seed survival in *M.*

*truncatula*, as well as for aphid resistance, both of which resulted in the successful discovery of new TFs that may function in these processes (Gao et al., 2010; Verdier et al., 2013). As a result, we used this qRT-PCR platform to identify TFs that respond to ABA signaling mediated by the nitrate transporter MtLATD/NIP (Yendrek et al., 2010; Bagchi et al., 2012).

Here we report the identification of TFs that have altered expression levels in *latd* mutant roots, and a core group of genes whose expression is restored to wild-type levels by ABA treatment. In addition, we demonstrated that ABA regulates a different set of TFs in *latd* mutants, indicating the important role that MtLATD/NIP plays in ABA-mediated transcriptional regulation. Finally, we isolated MtNSP2 from our TF profiling as a potential downstream TF under the regulation of both MtLATD/NIP and ABA. Then we showed that MtLATD/NIP is required for accurate temporal induction of MtNSP2 and MtNIN by cytokinin, and lastly, we demonstrated that MtLATD/NIP is required for TFs and their downstream gene expression in response to inoculation.

## **RESULTS**

### **TF Profiling Identifies MtLATD/NIP Regulated TFs**

To identify TFs that act downstream of MtLATD/NIP/NPF1.7, we took advantage of a qRT-PCR-based TF profiling platform developed for *M. truncatula* (Kakar et al., 2008). We chose 4 day-old *latd* roots for the TF profiling because we wanted to isolate TFs that are more directly responsive to changes in MtLATD/NIP function, rather than those that are associated with meristem arrest, which happens at about 21 days (Bright et al., 2005). At 4 days, the growth rate of *latd* mutants and wild-type primary root are similar (Bright et al., 2005). However, at this

early age, *latd* roots are already shorter, epidermal cell elongation is already reduced and ROS levels in *latd* roots are higher than in wild type (Zhang et al., 2014). To identify TFs that function in a pathway involving ABA and MtLATD/NIP, we harvested roots from 4-day old wild-type and *latd* plants that were grown continuously with or without 10  $\mu$ M ABA, and examined TF gene expression. At the time that we screened the qRT-PCR TF profiling platform, there were 1,084 TF targets, of which 855 were expressed at high enough levels to be detected in at least one of the four genotype/treatment combinations. We found that in *latd* roots grown on control medium, 20 TFs from 13 families showed a statistically significant difference (p-value <0.05) and greater than  $\pm 2$ -fold change ( $\pm 1$  for Log<sub>2</sub>fold change), compared to wild-type roots (Table 1 and 2, Fig. 1A). Of these, 11 TFs are up-regulated in *latd* roots, and 9 are down-regulated (Fig. 2, Table 1 and 2). The Cys2His2 (C<sub>2</sub>H<sub>2</sub>) zinc finger family has the largest number of members that exhibit altered expression levels in *latd* roots (Fig. 1, Table 1 and 2). However, the TF that is mis-expressed with the highest fold change in *latd* roots is a putative CCAAT-binding TF, *NUCLEAR FACTOR-Y SUBUNIT C1 (NF-YC1)* (GenBank accession number JQ918293.1; AC136503\_46.1 in Table 1, Fig. 2), which is expressed in wild-type roots, but is undetectable in *latd* roots. Interestingly, the expression of GRAS family TF *MtNSP2* (CR538722\_9.1 in Table 1), which has been shown to be one of the most important transcriptional regulators in nodulation signaling and plays a key role in mycorrhization (Kaló et al., 2005; Hirsch et al., 2009; Maillet et al., 2011), is much lower in *latd* mutant roots (with Log<sub>2</sub>Fold Change=-1.4, Table 1, Fig. 2C).

Since ABA rescues the *latd* root phenotype by decreasing ROS levels and increasing elongation of both primary and lateral roots (Liang et al., 2007; Zhang et al., 2014), we were interested in how the expression of TFs in *latd* roots treated with ABA differs from that of wild-type roots. To address this question, we compared TF gene expression between *latd* roots grown in the presence of ABA with wild-type plants grown on control medium. We expected that, since

ABA-treated *latd* plants are phenotypically similar to wild-type untreated plants (Liang et al., 2007), we would find that the patterns of TF gene expression in these two plants would be similar. Instead, we found that a total of 93 TFs show altered expression levels, with 22 down-regulated and 71 up-regulated TFs in *latd* roots grown on ABA as compared to untreated, wild-type plants (Fig. 2 and Supplemental Table S1), a huge increase over the 20 genes whose expression differs between wild-type and *latd* plants grown on control medium (Fig. 2 and Table 1 and 2). However, when we examine this set of 20 TFs that differ between untreated *latd* and wild-type roots, we find that the expression of 12 out of 20 of these TFs (60%) is restored by the addition of ABA (Fig. 2C, Table 1 and 2). Notably, the *NF-YCI* that is strongly repressed in *latd* mutant roots is restored to wild-type levels in ABA-treated *latd* roots (Table 1, Fig. 2C). These results suggest that ABA may rescue *latd* root elongation defects through restoring the expression of a core group of TFs.

### **Identification of TFs Regulated by ABA in *M. truncatula* Roots**

ABA plays an important role in mediating plant growth and stress responses. In *M. truncatula*, increasing ABA levels inhibits primary root growth, but induces lateral root formation (Liang and Harris, 2005; Liang et al., 2007). This regulation of root architecture by ABA is different from that of Arabidopsis and most other non-legumes, where ABA inhibits the formation of lateral roots (De Smet et al., 2003; Liang and Harris, 2005). This altered ABA response in legumes could be caused by a change in ABA signaling, but is more likely due to altered targets of ABA signaling, or perhaps altered responsiveness of the same targets to ABA. To identify *M. truncatula* root-expressed TFs regulated by ABA, we compared the gene expression profiles of TFs in wild-type whole roots treated with or without 10  $\mu$ M ABA for 4



days. Overall, we found that ABA regulates the expression of 79 TFs from 32 different TF families, with 63 TFs up-regulated by ABA, and 16 TFs down-regulated (Fig. 4 and Supplemental Table S2). Among the upregulated TFs, we found members of the ABI3, AP2/EREBP, bZIP, MYB and NAC families, which comprise many TFs known to play an important role in ABA signaling and ABA-mediated abiotic stress responses in various plant species (Fig. 3A). Down-regulated genes include members of the WRKY and GRAS families, including *MtNSP2* (Fig. 3A and Supplemental Table S2). Besides the known TF families that have been shown to play a role in ABA signaling genetically or biochemically, we also identified potential novel TF families that could function downstream of ABA. For example, another member of the CCAAT-binding TF, *NF-YC6* (accession number JQ918298.1), is up-regulated by ABA (Supplemental Table S2). TFs from the PHD family, WD-40 like family, and C2C2 zinc finger GATA TFs are also up-regulated by ABA treatment (Fig. 3A). None of these TF families from *M. truncatula* has been studied; the PHD TFs in Arabidopsis and barley have only been shown to play a role in pollen development and male sterility (Ito et al., 2007; Fernández Gómez and Wilson, 2014). Our TF profiling results suggest that these new TF family members might be important regulators downstream of ABA signaling and in mediating ABA responses in roots.

Gruber and colleagues have used the same high throughput qRT-PCR platform that we use here for identifying TFs differentially expressed under salt stress (Gruber et al., 2009). ABA mediates salt stress responses and tolerance, and many ABA-inducible genes are also up-regulated by high salt (Seki et al., 2002; Seki et al., 2002). We asked if any TF regulated by ABA identified in our study is also salt stress responsive. To do this, we compared our 79 ABA-regulated TFs with the data collected from Gruber et al., which identified 46 TFs whose expression levels were regulated by 1 hour of 100 mM NaCl treatment (Gruber et al., 2009). Interestingly, we found 7 of these salt-regulated TFs that are also regulated by ABA in our study

(Supplemental Fig. S1), suggesting these TFs may regulate ABA-mediated salt responses. However, the number of overlapping TFs regulated by both ABA and high salt is low (only about 6%), which could be due to the fact that the two studies have significant differences in plant growth conditions and the duration of treatments. Gruber and colleagues used liquid medium supplemented with NaCl on 2-week old plants, while we used 4-day old plants grown on solid medium with ABA added. In addition, our treatment was continuous, and theirs lasted one hour. Another reason for the different results could also be that transcriptional regulation of NaCl and ABA on TFs is largely different in *M. truncatula*. Three out of the 7 TFs were up-regulated by both high salt and ABA, and the remaining 4 TFs were oppositely regulated by the two treatments (Supplemental Fig. S1). Still, this comparison may allow us to identify TFs that may be regulated by both NaCl and ABA for future study.

#### **ABA restores the expression of a core group of TFs that are misexpressed in *latd* mutants**

MtLATD/NIP is required for ABA responses in seed germination inhibition and ABA-induced stomatal closure (Liang et al., 2007). *latd* root elongation defects, cell length defects and high root ROS levels can be rescued by exogenous ABA (Liang et al., 2007; Zhang et al., 2014). Since ABA can rescue the *latd* root defects, making the root grow and look very much like wild-type roots, we wondered whether the TF expression profiles of ABA-treated *latd* roots would resemble that of untreated wild-type roots. We found that the TF expression profile of ABA-treated *latd* roots was very different from that of wild-type roots (Fig. 2). However, we found that out of the 20 genes misexpressed in *latd* mutants, a core group of 12 TFs is restored to wild-type expression levels by ABA treatment (Fig. 2C). Thus, these genes likely include TFs that control root development pathways that cause the *latd* root defects.

### **MtLATD/NIP Alters TF Targets of ABA Signaling**

Since ABA regulated a similar number of genes in wild-type and *latd* roots (Fig. 4), we asked whether these transcriptional targets are the same in *latd* roots as in wild type. To address this question, we first compared the expression of TFs in *latd* treated with and without 10  $\mu$ M ABA. We found that there are 63 TFs total, from 28 different families that exhibit significantly altered expression levels by ABA treatment (Fig. 5 and Supplemental Table S3). Among these TFs, the expression of 60 TFs is up-regulated by ABA and that of only 3 TFs is down-regulated (Fig. 5 and Supplemental Table S3). As in wild-type roots, we also isolated TFs from ABI3, AP2/EREBP, MYB and NAC families, typical ABA-responsive TF families (Fig. 3B). However, there are some differences in the TF families regulated by ABA. For example, the BTB/POZ domain and bZIP TF families each have a member up-regulated by ABA in wild type (Fig. 3A), but this regulation is missing in *latd* roots (Fig. 3B). BTB/POZ domain containing proteins have been shown to play a role in disease resistance and plant development in Arabidopsis, and they can interact with AP2/ERF family TFs for transcriptional control (Boyle et al., 2009; Weber and Hellmann, 2009; Chen et al., 2013). Moreover, only 2 GRAS family TFs and 4 NAC family TFs were regulated by ABA in *latd* roots, while 4 and 6 were regulated in wild-type roots, respectively (Fig. 3A and B). Therefore, the transcriptional regulation by ABA on different TF families in *latd* roots is partially intact, but with significant differences compared to wild type.

We then compared the TFs regulated by ABA in wild-type and *latd* roots. Interestingly, it turned out that of all TFs regulated by ABA in both genotypes, only 26 out of the 116 TFs (22.4%) are regulated by ABA in both genotypes (Fig. 4A and B, Supplemental Table S4). Only 24 out of 99 TFs (24.2%) are up-regulated by ABA in both wild-type and *latd* mutants (Fig. 4A);

2 out of 17 TFs (11.8%) are down-regulated in both genotypes by ABA (Fig. 4B, Supplemental Table S4). This indicates that the majority of TFs that are regulated by ABA are different in wild-type and *latd* roots; in other words, the *latd* mutation significantly changes ABA regulation of TF expression. Notably, MtNSP2 is down-regulated in both wild-type and *latd* roots, although the inhibitory effect of ABA appears to be stronger in the *latd* mutant (Supplemental Table S3).

Again we compared the TFs regulated by ABA in *latd* mutants with salt stress-regulated TFs from Gruber et al. (Gruber et al., 2009). We found all 7 TFs that were regulated both by salt and by ABA in wild type are also present in ABA-regulated TFs in *latd* roots, with 2 TFs (MtNAC969 and MtbHLH85) that are unique for ABA induction only in *latd* mutants but did not show any change in wild-type ABA-treated roots (Supplemental Fig. S1). MtNAC969 negatively regulates salt stress responses as well as nodule senescence (De Zélicourt et al., 2012). Its expression is induced by ABA rapidly, by 3 hours, and also by nitrate in two days (De Zélicourt et al., 2012). This could explain why we did not see NAC969 being induced in wild-type roots by our ABA treatment, since it is a much longer, continuous application. However, the expression of NAC969 is up-regulated in *latd* mutants by a 4-day continuous ABA treatment, again indicating that ABA's regulation in *latd* roots may be either delayed or prolonged. NAC969 also plays a role in regulating root architecture; overexpressing NAC969 leads to a shorter primary root and decreased lateral root density, while reducing its expression results in increased LR density, but has no effect on primary root growth (De Zélicourt et al., 2012). In *latd* mutants, ABA increases primary root and LR elongation, which does not seem to correlate with the expression profile of NAC969 being induced by ABA in *latd* roots.

### **Regulation of MtLATD/NIP on TF Expression is Dependent on the Presence of ABA**

Our initial analysis identified 20 TFs that exhibit altered gene expression when the LATD/NIP gene is disrupted (Table 1 and 2, Fig. 1A). We then asked whether the application of 10  $\mu$ M ABA changes the TFs regulated by MtLATD/NIP. To do this, we compared TF gene expression in wild-type and *latd* roots both treated with ABA. The expression of 30 TF genes showed a significant difference between wild-type root and *latd* root both grown on ABA, with 21 up-regulated and 9 down-regulated, from 18 different TF families (Fig. 1B, Supplemental Table S5). Then we wondered whether the application of ABA changes the TFs that are regulated by MtLATD/NIP under control conditions. To do this, we compared TFs that have altered expression levels in *latd* mutants relative to wild-type roots at the same growth conditions, i.e. with and without ABA. Surprisingly, we only found two TFs regulated by MtLATD/NIP both with and without ABA (Fig. 5A and B). Only the expression of 1 TF, MtNSP2, is down-regulated in *latd* mutants both with and without ABA, while a C<sub>2</sub>H<sub>2</sub> zinc finger protein with uncharacterized function is up-regulated in *latd* roots under both conditions (Table 2 and Supplemental Table S5). Overall, among the TFs that exhibit altered expression levels in wild-type and *latd* root grown with 0  $\mu$ M and 10  $\mu$ M ABA, only 4.17% are regulated by MtLATD/NIP (Fig. 5A and B). This indicates that the application of 10  $\mu$ M ABA changes the TFs regulated by MtLATD/NIP almost completely, and that the regulation on TF expression by MtLATD/NIP can be reprogrammed by the addition of ABA.

### **MtNSP2 Expression is Regulated by MtLATD/NIP, ABA and Nitrate**

One of most surprising observations from our TF profiling results is that the key symbiotic regulator, MtNSP2, shows different expression levels in all of our comparisons. That is, MtNSP2 is down-regulated in *latd* roots compared to wild-type, and ABA inhibits its

expression in both wild-type and *latd*, but with different fold changes (Fig. 6A). This initial observation has lead us to ask, could this well-known TF in Nod factor signaling have a novel function in regulating root growth and development mediated by ABA? To address this question, we took advantage of the *nsp2-2* mutant, which contains a deletion in the functional GRAS domain (Kaló et al., 2005). We grew *nsp2-2* mutants with 10  $\mu$ M ABA and observed the root phenotypes compared with wild-type plants. *nsp2-2* mutants grown on 10  $\mu$ M ABA have shorter primary roots, and more lateral roots, just as wild-type plants do (Fig. 6B and C). We also checked the expression of the ABA-responsive gene *MtRD22* (Ding et al., 2008), which is induced in *nsp2-2* mutants by ABA, but with a higher fold induction than in wild type (Fig. 6D). It is possible that *nsp2-2* mutants may have increased sensitivity to ABA, but this needs to be confirmed by checking *nsp2-2* mutants phenotypes under lower ABA concentrations.

A microarray study done by Liu. et al. on 7 day-old uninoculated *nsp2-2* roots tissue has provided some evidence of altered gene expression pattern related to ABA metabolism and signaling (Liu et al., 2011). We verified the expression of some of these genes using whole root tissue grown continuously with and without the exogenous ABA. We confirmed that the expression of two ABA 8'-hydroxylases identified by Liu and colleagues (TC165592 and TC177719) (Liu et al., 2011), which promote the irreversible degradation of ABA, are down-regulated in *nsp2-2* mutant roots (Fig. 7A and B). On the other hand, the expression of an ABA biosynthetic enzyme NINE-cis-EPOXYLCAROTENOID DIOXYGENASE 4 (NCED4) (CX549419) is also down-regulated in *nsp2-2* mutant roots (Fig. 7C). Interestingly, ABA down-regulates the expression of both ABA 8'-HYDROXYLASE (TC165592) and *NCED4* in wild type, but this inhibition is abolished in *nsp2-2* mutants. In addition, a short chain alcohol dehydrogenase (CX549464), with homology with Arabidopsis *ABA2* (Liu et al., 2011), which

catalyzes one of the key steps in ABA *de novo* biosynthesis from xanthoxin to abscisic aldehyde, is down-regulated in *nsp2-2* mutants. A *LEA4* gene (TC147896), which has homology with the ABA-responsive Arabidopsis LATE EMBRYOGENESIS ABUNDANT 4-5 (*LEA4-5*)(Olvera-Carrillo et al., 2010; Liu et al., 2011), is down-regulated in *nsp2-2* mutants. Notably, as mentioned above, all four ABA metabolic and responsive genes, *ABA 8'-HYDROXYLASE*, *NCED4*, *CX549464* and *LEA4* were all down-regulated in the *nsp1* mutant as well. NSP1 is another GRAS TF that forms heterodimers with NSP2 during nodulation signaling and binds to down-stream signaling genes (Hirsch et al., 2009). The fact that four ABA-related gene expression are down-regulated in both *nsp2* and *nsp1* mutants may indicate that NSP2 plays a role in mediating ABA homeostasis and signaling, likely via the interaction with NSP1.

Since MtLATD/NIP is a nitrate transporter (Yendrek et al., 2010), and disruption of this gene leads to decreased expression levels of *NSP2*, we wondered if nitrate could also affect *NSP2* expression. To test this, we transferred 7 day-old plants to 10  $\mu$ M ABA and 10 mM KNO<sub>3</sub> for 3 days. We found that both a 3-day ABA and KNO<sub>3</sub> treatment are able to repress NSP2 expression, although ABA has a stronger inhibitory effect (Fig. 8A). *NSP2* expression in *latd* mutant roots showed similar responses to ABA and nitrate as in wild type (Fig. 8A). Since nitrate regulates NSP2 expression, and it has been shown that the expression of nitrate reductases (NR) was down-regulated in the Nod factor susceptible zone of *nsp2-2* mutants (Liu et al., 2011), we wondered whether *nsp2-2* mutants might have altered nitrate responses. To test this, we grew the *nsp2-2* mutant and wild type on growth medium supplemented with different concentrations of KNO<sub>3</sub> then examined root growth responses. It has been shown in Arabidopsis that systemic nitrate treatment leads to inhibition of lateral root growth, while local nitrate with low concentrations (<1 mM) can stimulate lateral root elongation. We tested three different concentrations of nitrate, 250  $\mu$ M, 1mM and 10mM and used the same concentrations of KCl as a control. We found 1 mM

nitrate stimulates wild-type primary root growth and lateral root density, but does not affect *nsp2-2* mutants at this concentration (Fig. 9A). In contrast, low concentration of nitrate (250  $\mu$ M), can stimulate lateral root density and total lateral root length per cm of primary root in *nsp2-2* mutants, but wild-type plants do not respond to nitrate at this concentration (Fig. 9B and C). One mM nitrate can also stimulate total lateral root length per cm primary root in both wild-type and *nsp2-2* mutants (Fig. 9C). In summary, we did not observe a significant altered response to nitrate in *nsp2-2* mutant, but *nsp2-2* mutants do exhibit increased sensitivity to the stimulation effect of systemically applied nitrate at low concentration on lateral root development.

Recently, a microRNA, mir171h has been shown to target the MtNSP2 transcript and negatively regulate its expression during mycorrhizal colonization (Devers et al., 2011; Lauressergues et al., 2012). We asked if this microRNA is also regulated by MtLATD/NIP, ABA and nitrate. Again we checked the expression of Mt*PREMIR171h* in plants treated with ABA or nitrate for 3 days. We found that in *latd* mutant roots, the expression of Mt*PREMIR171h* is higher than wild type, despite the fact of MtNSP2 expression in *latd* roots is already low (Fig. 8B). ABA also represses its expression in wild-type roots, possibly due to a negative feedback loop. However, nitrate can up-regulate the expression of Mt*PREMIR171h*, both in wild-type and *latd* mutant roots (Fig. 8B), which is consistent with the observation that nitrate down-regulates MtNSP2 expression (Fig. 8A). In summary, our results show that both MtNSP2 and Mt*PREMIR171h* expression is regulated by MtLATD/NIP, ABA and nitrate. The fact that nitrate up-regulates the expression of Mt*PREMIR171h* provides a possible mechanism for the way in which nitrate represses MtNSP2 expression.



## MtLATD/NIP Mediates Nodulation and Cytokinin Signaling Crosstalk

MtNSP2 is an essential TF that is required for nodulation signaling, and it is also under tight regulation by cytokinin (Ariel et al., 2012). Cytokinin plays an important role in nodule organogenesis by inducing cell division in the inner cortex (Oldroyd and Downie, 2008). The cytokinin signaling regulator, Type-B Responsive Regulator 1 (RR1), can directly bind to the promoter of *NSP2* and induce its expression in response to cytokinin (Ariel et al., 2012). Since MtLATD/NIP regulates the expression of *NSP2*, and *latd* mutant roots exhibit Nod<sup>-</sup> phenotypes, we asked whether MtLATD/NIP is involved in cytokinin responses. To do this, we treated wild-type and *latd* mutants with a short-term exogenous cytokinin treatment, benzyl amino purine (BAP) for 1 hour and 3 hours, and then checked cytokinin-responsive gene expression. The expression of cytokinin receptor MtCRE1 and a Type-A RR MtRR4 is rapidly induced by BAP, in both wild-type and *latd* roots, with almost exactly the same pattern (Fig. 10D and F). In addition, MtRR1 expression did not show significant changes during this time course (Fig. 10E). The *NSP2* gene is highly induced by BAP in wild-type roots at 1 hour, and its expression declined somewhat at the 3 hour time point (Fig. 10A). In *latd* mutants, however, *NSP2* is not induced by cytokinin. We also checked MtNIN expression, which is regulated by NSP2 during Nod factor signaling. We found that *NIN* expression can be induced by cytokinin in *latd* roots at 1 hour as in wild type; however, the pattern of expression induction is different in *latd* roots. In wild-type, *NIN* expression continues increasing to an even higher level at the 3 hour time point, while in the *latd* mutant, *NIN* expression at 3 hour declined to levels lower than that at 1 hour (Fig. 10B). another gene downstream of NSP2 in the cytokinin response pathway is one of the CLAVATA3/embryo-surrounding region (CLE) peptides, MtCLE13, which regulates autoinhibition of nodulation (Mortier et al., 2010). We observed a strong induction of MtCLE13 by cytokinin at the 3 hour time point in wild-type roots with over 188-fold induction, but this

induction happens at very low levels in *latd* roots, although there is a significant increase, about 18-fold (Fig. 10C). Thus, these findings suggest that when *MtLATD/NIP* gene is disrupted, a branch of cytokinin signaling is intact, involving the cytokinin receptor CRE1, and Responsive Regulators RR4 and RR1 (Fig.10G). However, the cytokinin-responsive genes that are involved in early Nod factor signaling, NSP2, NIN and MtCLE13, require a functional MtLATD/NIP for cytokinin induction (Fig. 10G). Ariel and colleagues have recently shown that RR1 binds to the promoter of both NSP2 and RR4 and turn on their expression in response to cytokinin (Ariel et al., 2012). As a result, MtLATD/NIP is likely to mediate cytokinin-inducible *NSP2* expression downstream of RR1, although we could not rule out the possibility of MtLATD/NIP affecting RR1 binding to the promoter of NSP2. Since NSP2, NIN and CLE13 are all important regulators in nodulation, it is reasonable to speculate that MtLATD/NIP plays a role in regulating early nodulation gene expression in response to *S. meliloti* inoculation.

To further test this possibility, we checked early nodulation gene expression after inoculation. Wild-type plants inoculated with *Sinorhizobium meliloti* Rm1021 exhibit strong induction of *NIN* and Mt*CLE13* expression at 1 day-post-inoculation (dpi), and a slight increase in *NSP2* expression, about 1.6-fold (Fig. 11A). However, in *latd* mutants, the induction of *NIN* was not observed at 1 dpi, with only a slight increase at 3 dpi (Fig. 11B). The induction of *CLE13* in response to inoculation is completely abolished in *latd* mutant roots, and *NSP2* expression remains unchanged as well (Fig. 11C). A downstream target of NSP2, *EARLY NODULIN 11* (*ENOD11*) (Hirsch et al., 2009), which has been used as a reporter gene for Nod factor signaling in the root epidermis (Charron et al., 2004), is also highly induced upon inoculation in wild-type roots, with a over 100-fold increase at 1 dpi; we did not observe its induction in *latd* roots until 2 dpi, but even then it still remains at a much lower level compared to that in wild type at this time point (Fig. 11D). MtERN1, an AP2/ERF TF in Nod factor signaling involved in IT formation,

functions downstream of MtNSP2 and cytokinin receptor MtCRE1, but also bind to the MtENOD11 promoter at a separate region from that of MtNSP2 (Andriankaja et al., 2007; Middleton et al., 2007; Cerri et al., 2012). It has been shown that *MtERN1* expression is induced by *S. meliloti* inoculation, and the spatial pattern of MtERN1 is dynamic: it is observed in root hair cells during the preinfection stage, then in the root hairs during infection, and also in outer cortex cells in later stages (Cerri et al., 2012). We also observed a strong induction of *MtERN1* expression during early preinfection stage in wild-type roots at 1 dpi and 2 dpi (Fig. 11E). However, in *latd* mutants inoculated with *S. meliloti*, we did not observe any significant induction of *MtERN1* expression until 2 dpi, and the expression level is much lower than in wild-type (Fig. 11E). Together, these results indicate that MtLATD/NIP is required for the transcriptional induction of many important TFs in the Nod factor signaling pathway, such as MtERN1 and MtNIN. MtLATD/NIP is also necessary for the induction of MtENOD11 and MtCLE13, which are expressed in epidermis and cortex respectively. Therefore, MtLATD/NIP plays a role in mediating the crosstalk between Nod factor and cytokinin signaling in nodule organogenesis in both the epidermis and cortex pathways (Fig. 11F).

## DISCUSSION

In this study we identified TFs that have altered expression levels in *latd* mutant roots, which indicates a role for nitrate transporter MtLATD/NIP in regulating the expression of TFs. Exogenous ABA treatment can rescue the expression of 60% of the TFs in *latd* roots that have altered expression levels, providing important downstream targets of ABA in regulating root growth. More importantly, since ABA rescues *latd* root defects, it is possible that these TFs are major regulatory nodes in MtLATD/NIP and ABA signaling. We identified TF families whose

expression is responsive to ABA, and TFs that could function in an ABA-salt stress pathway. MtNSP2, which was isolated in our TF profiling as being regulated transcriptionally by both MtLATD/NIP and ABA, also plays a role in ABA and nitrate responses in roots. Lastly, we showed that MtLATD/NIP is required for inducing the expression of genes functioning in both cytokinin and nodulation signaling via regulating MtNSP2, suggesting that MtLATD/NIP functions at a regulatory node upstream of MtNSP2 in transducing Nod factor signaling and coordinating the nodulation responses in both epidermis and cortex. These findings have revealed components of a pathway downstream of MtLATD/NIP that controls TF gene expression, as well as a central role in coordinating rhizobia infection and cortical cell division in nodule formation.

#### **Nitrate Transporter MtLATD/NIP Influences ABA-Regulated TF Gene Expression**

Here we show that the TFs whose expression is regulated by ABA are very different in *latd* mutant roots when compared with those in wild-type roots (Fig. 4A and B). This finding indicates that the regulation of ABA on TF gene expression is dependent on MtLATD/NIP; without functional MtLATD/NIP, more than 80% of downstream target TFs in ABA signaling change. The *latd* mutant is insensitive to ABA for ABA-induced stomatal closure and seed germination (Liang et al., 2007), indicating that MtLATD/NIP is required for ABA responses. Our TF profiling results show that MtLATD/NIP is also crucial in mediating ABA-responsive TF expression in roots, which provides further evidence of its role in different aspects of ABA responses across different tissue types. Moreover, among the 20 TFs showing altered expression levels in *latd* roots, the expression of 60% of these TFs can be restored by applying 10  $\mu$ M ABA (Fig. 2, Table 1 and 2). This finding correlates well with the fact that exogenous ABA can rescue *latd* root growth defects (Liang et al., 2007), and these specific TFs whose expression is rescued

by ABA may be the key TFs downstream of ABA signaling mediated by MtLATD/NIP whose misexpression is responsible for the *latd* root defects.

We have recently shown that the homeostasis of ROS in *latd* roots is disrupted, and ABA rescues primary root and LR elongation in *latd* mutants via decreasing ROS levels (Zhang et al., 2014). Thus, it is possible that some of the TFs with altered expression levels in *latd* roots are also ROS-regulated, and that MtLATD/NIP mediates ABA regulation of TF transcription via modulating ROS levels. ROS are signaling molecules that play an important role in ABA signal transduction in both guard cells and roots (Swanson and Gilroy, 2010). The fact that MtLATD/NIP modulates ROS levels, although the mechanism is not completely elucidated, may be one way of how MtLATD/NIP affects downstream TF gene expression. ROS can directly induce alterations of protein kinases and transcription factors, in both animals and plants (Adler et al., 1999). Expression of many TFs have been shown to be ROS-responsive, including members of the bHLH, WRKY, NAC, MADS-box, DREB and C2H2 zinc finger TF families (Vanderauwera et al., 2005; Gadjev et al., 2006; Mittler et al., 2006; Wu et al., 2012). In *latd* mutants, the C2H2 zinc finger family has the largest number of members that exhibit altered expression levels, with three up-regulated and two down-regulated (Table 1 and 2) and ABA restores the expression of three out of these five C2H2 zinc finger TFs (Table 1 and 2). It will be interesting to test if these C2H2 TFs are ROS-regulated, and whether they function in ABA and ROS signaling in root elongation.

MtLATD/NIP has been shown to transport nitrate in *Xenopus* oocytes at a low external nitrate concentrations (in the  $\mu\text{M}$  range), therefore it is a high affinity nitrate transporter (Bagchi et al., 2012). However, nitrate uptake in the *nip-1* mutants monitored by nitrate depletion from the media did not exhibit any difference from wild-type seedlings (Bagchi et al., 2012), suggesting

the presence of other nitrate transporters that are functionally redundant. Thus the *latd* mutant phenotype is probably not due to the lack of nitrate transport activity of MtLATD/NIP, since the *latd* mutants show strong phenotypes even when there is no nitrate present and expression of another known nitrate transporter cannot complement the *latd* phenotype (Bright et al., 2005). It is reasonable to speculate that other than transporting nitrate, a more important signaling role of MtLATD/NIP exists. It could be mediating ABA signaling, either via the crosstalk between ABA and nitrate, or via an unknown mechanism. This could explain the significant change in TF gene expression regulated by ABA when MtLATD/NIP gene is disrupted. MtLATD/NIP belongs to the NRT1/PTR family (now called NPF) (Léran et al., 2014), where AtNRT1.1 also resides, although these two genes are not very closely related (Yendrek et al., 2010). AtNRT1.1 has been extensively studied, and it functions as a dual affinity nitrate transporter, but also as a nitrate sensor. Moreover, AtNRT1.1 can also transport auxin when the external nitrate concentration is low, while the presence of high concentration of nitrate can inhibit its ability to transport auxin shootward, resulting in more auxin accumulation in the root tip and promoting the outgrowth of LR<sub>s</sub> (Krouk et al., 2010). Similar to AtNRT1.1, MtLATD/NIP is expressed in the root tips of primary and lateral roots, suggesting its possible function in sensing environmental signals such as nutrient patches in the soil. So far there is no evidence that MtLATD/NIP can transport auxin as AtNRT1.1 does, but MtLATD/NIP could also be a nitrate sensor. Moreover, the expression of AtNRT1.1 is induced by nitrate and auxin (Tsay et al., 1993; Guo et al., 2002), but this is not the case for MtLATD/NIP, whose expression is strongly decreased by auxin, and not affected by nitrate (Yendrek et al., 2010). However, the fact that disruption in the MtLATD/NIP gene strongly affects ABA signaling suggests a tight link between nitrate and ABA signaling mediated by MtLATD/NIP. It is possible that MtLATD/NIP functions as a nitrate sensor and affects ABA accumulation, transport or signaling, and this may depend on the nitrate concentrations present around the root tips in the growth environment. In this case, it would be worth investigating the

spatial expression pattern of the 12 TFs that have altered expression levels in *latd* roots but are rescued by ABA; the root tip-expressed TFs may be possible downstream regulatory components in mediating root development in response to local nitrate signals sensed by MtLATD/NIP.

ABA and nitrate signaling are interconnected, and ABA plays a central role in mediating the effect of nitrate on LR branching (Signora et al., 2001). In Arabidopsis, LR formation is inhibited systemically by high concentrations of nitrate (10 and 50 mM), and this effect is developmentally dependent, where LR elongation is affected immediately after emergence. *abi4* mutants are much less sensitive to this inhibitory effect of nitrate, as well as some ABA-biosynthesis mutants (Signora et al., 2001), indicating that ABA levels and signaling are required for root architecture high nitrate responses. *abi1*, *abi2* and *abi5* mutants seem to have altered but complicated responses to high nitrate in LR development, which indicates that novel signaling components may play a major role in the cross-talk between ABA and high nitrate in the inhibition of LR development. Localized low concentrations of nitrate stimulates LR formation, mainly during the elongation phase (Zhang et al., 1999). This stimulatory effect is reduced in *aba1* and most *abi* mutants (Signora et al., 2001), indicating a positive role for ABA in regulating localized low nitrate-induced LR elongation. Notably, different concentrations of nitrate tested (0.1, 1, 10, 50 mM) did not lead to any obvious changes in primary root growth in Arabidopsis wild-type seedlings (Signora et al., 2001). In *M. truncatula*, systemic nitrate treatment at 1 and 10 mM decreased primary root length but had no effect on LR density, and nitrate at these concentrations did not affect or rescue root growth defects in *latd* mutants (Yendrek et al., 2010). However, 5 mM nitrate seems to partially rescue LR elongation in *nip-1* and *nip-3* mutants, two other alleles of MtLATD/NIP (Bagchi et al., 2012). LR elongation in wild-type *M. truncatula* can be stimulated by systemically applied nitrate at 250  $\mu$ M and 5 mM, but the stimulatory effect at 250  $\mu$ M nitrate is abolished in the *nip-1* mutant (Bagchi et al., 2012). Explanations of this

observation could be that MtLATD/NIP mediates uptake or sensing low concentrations of nitrate, but not high concentrations. In this study, we observed the inhibitory effect of 1 mM nitrate on wild-type primary root elongation, and the stimulatory effect of nitrate on LR elongation was found only at the 1 mM concentration, not at 250  $\mu$ M, which could be due to different plant growth methods and different developmental stages when phenotype was examined (Fig. 9). It would be interesting to check if the expression of any TFs is also regulated by nitrate, ABA and MtLATD/NIP. Clearly the effect of systemically applied nitrate in root growth in *M. truncatula* is different from that in Arabidopsis; whether the localized nitrate patches also have different effect in *M. truncatula* LR development and whether it is mediated by MtLATD/NIP and its downstream TFs would be interesting to investigate. MtLATD/NIP facilitates nitrate uptake when expressed in *Xenopus* oocytes, and overexpressing Arabidopsis NRT1.1 under EF1 $\alpha$  promoter can partially rescue *nip-1* LR elongation, but not the nodulation phenotype (Bagchi et al., 2012). AtNRT1.1 is a dual affinity nitrate transporter that contains a Thr101, which serves as a phosphorylation site of calcineurin-like protein (CBL)-interacting protein kinase 23 (CIPK23) in response to different concentrations of nitrate (Liu and Tsay, 2003; Ho et al., 2009). AtNRT1.1 can be highly phosphorylated under low nitrate concentrations (in  $\mu$ M range), and this phosphorylation switches the low affinity homodimeric transporter to a high affinity monomeric nitrate transporter (Liu and Tsay, 2003; Sun et al., 2014). In this case, AtNRT1.1 is both a nitrate transporter and a nitrate sensor that mediates downstream nitrate responses, such as gene expression and root branching. Whether such post-translational regulation of MtLATD/NIP exists is unknown, it would be worthwhile to explore if there is any protein kinase in ABA signaling phosphorylates MtLATD/NIP and regulates its activity.



### Novel TFs that Are Regulated by ABA in *M. truncatula*

We identified 79 TFs from 32 different families whose expression is regulated by 4-day continuous 10  $\mu$ M ABA treatment in wild-type *M. truncatula* roots (Fig. 3 and Supplemental Table S2). Many of the TF members are from TF families that have been shown to be ABA-regulated in other plant species, such as NAC, bHLH, MYB, WRKY, AP2/EREBP and many others, but we also found TF families that have not been identified as ABA-responsive (Fig. 3). In contrast to studies in *Arabidopsis* and maize, we did not find many bZIP TF family members whose expression is regulated by ABA (Fig. 3). bZIP TFs have been shown to be in the core ABA signaling pathway and can be activated directly by SnRK2 (Nakashima and Yamaguchi-Shinozaki, 2013; Marco Llorca et al., 2014). The fact that we did not identify many bZIP TFs in our analysis is probably due to the fact that many bZIP TFs are rapidly activated in response to ABA, but our approach was on a much longer, 4-day ABA treatment.

There are four GRAS family TFs that exhibit altered expression in response to ABA treatment in wild-type roots, all of which are down-regulated, including MtNSP2. These GRAS TFs do not contain the DELLA domain, so they are not the DELLA proteins that function in gibberellin signaling, which works antagonistically to ABA signaling in the regulation of plant growth and development (Golldack et al., 2013). It will be interesting to investigate the role of these GRAS TFs in ABA signaling. We also found that MtNSP2 is down-regulated in *latd* mutant roots, suggesting that the wild-type function of MtLATD/NIP has a positive role in MtNSP2 expression levels. Since ABA can still down-regulate MtLATD/NIP expression in *latd* to an even lower level (Fig. 6A), it seems that ABA and MtLATD/NIP can both regulate MtNSP2 expression, but independently, in separate pathways. We also found that a 3-day 10 mM nitrate treatment can decrease MtNSP2 expression in both wild-type and *latd* roots, just as ABA does

(Fig. 8A). MtNSP2 functions downstream of Nod factor signaling and is required for nodule formation (Kaló et al., 2005). It is possible that the inhibitory effect of ABA and high concentrations of nitrate on MtNSP2 expression is due to the fact that both of them negatively regulate nodulation (Ding et al., 2008; Mohd-Radzman et al., 2013). As discussed above, MtLATD/NIP may regulate root architecture by acting as a nitrate sensor, besides its role of being a high-affinity nitrate transporter in the root tips, and this regulation is likely to correlate with ABA signaling and responses. It is possible that MtLATD/NIP also functions in nodulation as a nitrate sensor. *latd* mutants do not form functional nodules, but bacterial infection can happen, but IT progression and bacterial release are to a much less extent. It is clear that MtLATD/NIP is required for nodulation, but since it transports nitrate, a negative regulator of nodulation, an alternative explanation would be that MtLATD/NIP must have a novel function that is not just simply transporting nitrate, but positively regulate nodulation. Maybe MtLATD/NIP is required for maintaining basal expression level of MtNSP2, which is an important TF required for inducing downstream gene expression for nodule formation. A recent study looking at the transcriptional regulation by ROS in nodulation and found that inoculating root with blocked ROS production using diphenylene iodonium (DPI) lead to decreased MtNSP2 expression (Andrio et al., 2013). We have shown that ROS levels are increased in *latd* roots (Zhang et al., 2014), where MtNSP2 expression is decreased, and we did not observe any significant changes in MtNSP2 expression in *latd* root treated with cytokinin or inoculated *latd* roots (Fig. 10 and Fig. 11). It is possible that MtNSP2 is also responsive to ROS levels and MtLATD/NIP regulates MtNSP2 expression via its role in ROS homeostasis.

### **Can MtNSP2 Coordinate Nitrate, ABA and Nodulation Signaling in Root Development?**

Since legumes form two kinds of lateral organs on the roots, LR and nodules, the regulation of the architecture of whole root system is more complicated, and the way in which nodule development is integrated into the developmental program controlling root development is intriguing (Desbrosses and Stougaard, 2011). There are several lines of evidence that nodule formation and overall root architecture are linked. Inoculating legume roots with rhizobia appears to have an inhibitory effect on host plant root development (Desbrosses and Stougaard, 2011). In *Lotus japonicas*, it has been shown that inoculated roots have a slower growth rate compared to uninoculated roots, and this inhibitory effect is exaggerated in the hypernodulating *har1* mutants, which are defective in autoregulation of nodulation (AON) (Wopereis et al., 2000). Since legume plants have to supply a carbon source to rhizobia for nitrogen fixation, it is important to limit the number or the density of nodules being formed. Notably, uninoculated *har1* mutants have a short root system with an increased number of LR primordia and emerged LRs (Wopereis et al., 2000). It is possible that the short root phenotype of *har1* is due to an imbalance of the two types of lateral organs, and thus it is crucial to control the formation of nodules and lateral roots, to achieve the balance of nitrogen fixation and plant growth. Such a balance between LR and nodule formation has been noticed in different wild-type strains and has led to the hypothesis that the plant coordinates these two processes (Nutman, 1948).

Low concentrations of nitrate (in the  $\mu\text{M}$  range) can stimulate LR formation in *Arabidopsis* (Zhang et al., 1999). We found that 1 mM nitrate stimulates LR density and elongation in *M. truncatula* wild-type plants (Fig. 9). Our results also showed that low concentrations of nitrate (250  $\mu\text{M}$ ) can stimulate LR density and total LR elongation in *nsp2-2* mutants, but did not affect wild-type LR formation (Fig. 9), suggesting when the MtNSP2 gene is disrupted, LR formation is more sensitive to the effect of low nitrate. In other words, MtNSP2 may function as a negative regulator of LR formation in low nitrate conditions. Since MtNSP2 is

required in nodulation, it is possible that at low nitrate concentrations, MtNSP2 inhibits LR formation, but stimulates nodulation signaling to initiate nodule organogenesis. In such way, MtNSP2 could help to balance the formation of these two types of lateral root organs, which is important for the growth of whole root system. Moreover, MtLATD/NIP is a root tip-expressed high-affinity nitrate transporter (Yendrek et al., 2010; Bagchi et al., 2012); if it also functions as a nitrate sensor responsive to low concentrations of nitrate present in the environment, maybe MtLATD/NIP can perceive and transduce the low nitrate signal at the LR tip, then further positively modulate MtNSP2 levels to turn on nodulation signaling. To test this possibility, it is important to investigate whether MtLATD/NIP expression or activity in *M. truncatula* roots, as well that of MtNSP2, is regulated by low concentrations of nitrate.

### **MtLATD/NIP Functions in Cytokinin and Nodulation Crosstalk**

Nodule organogenesis is a complex process involving coordination between bacterial infection and cell division (Oldroyd and Downie, 2008). *latd* mutants have been shown to be defective in both processes: the nodules on *latd* roots are small and white, with no nitrogen fixation activity (Bright et al., 2005). Bacterial infection is incomplete, a few IT reach the nodule primordia, although these ITs barely branch (Bright et al., 2005). Rhizobial differentiation is also blocked at later stages in *latd* mutants, most likely since nodule development is delayed (Bright et al., 2005). Since we identified MtNSP2 in our TF profiling as being regulated by MtLATD/NIP and ABA transcriptionally, this led us to examine more closely the role of MtLATD/NIP in Nod factor signaling at the gene expression level. Mt*ENOD11*, induced within the first 6 hours after Rhizobium inoculation in the epidermis (Marsh et al., 2007), is not induced even by 2 dpi in *latd* roots (Fig. 10). We observed the same pattern for Mt*ERN1* expression in *latd*, which does not

show induction until 2 dpi, and then the induction is still very weak compared to wild type (Fig. 11). *MtERN1* expression is rapidly induced by Nod factor, and is under the regulation of MtNSP2 and MtNSP1 (Hirsch et al., 2009; Cerri et al., 2012). The MtERN1 protein is localized to the epidermal cells in the Nod factor susceptible zone, and later to the outer cortical cells. *ern1* mutants have defects not in the number of infection foci, but in the progression of ITs, which pinpoints MtERN1 to be necessary in positively regulating the development of the IT in the epidermis and outer cortex (Middleton et al., 2007; Cerri et al., 2012). The fact that both MtERN1 and the MtNSP1, MtNSP2 complex bind to the promoter of *MtENOD11* and contribute to its full induction (Cerri et al., 2012), and that *MtERN1* and *MtNSP2* levels remain very low in *latd* mutants, likely explain the delayed and lower induction of *MtENOD11* in *latd* roots. The number of infection events during early stages of nodulation (0-3 dpi) is lower in *latd* (Bright et al., 2005), and correlates well with the expression profile of genes involved in pre-infection and the progression of infection identified in our study. These observations also suggest that MtLATD/NIP functions early during nodulation in the epidermis and is required for the full induction of *MtERN1* and *MtENOD11* by MtNSP2. MtNIN, another TF functioning downstream of  $\text{Ca}^{2+}$  spiking and MtNSP2, is required for both nodule organogenesis in cortex and rhizobial infection in the epidermis (Marsh et al., 2007). We did observe weak induction of MtNIN expression in inoculated *latd* roots, but again is delayed and the expression level remained very low (Fig. 11). MtCLE13, whose expression is induced specifically in the inner cortical cells going through cell division during nodulation (Mortier et al., 2010), is not induced in *latd* mutants (Fig. 11), suggesting that either fewer cells are dividing in response to inoculation, or perhaps the perception of a cell division signal is defective. These findings indicate that MtLATD/NIP is required for the induction of genes involved in both epidermal and cortical signaling pathway in response to inoculation, and that MtLATD/NIP functions upstream of MtNSP2.

Many nodulation genes are also cytokinin-inducible, since cytokinin functions as a signal in the inner cortex to induce cell division in response to the rhizobial Nod factor signal in order to form nodule primordia. We found that the expression of MtNIN and cytokinin receptor MtCRE1, as well as a Type-A RR, RR4, can be induced in *latd* roots treated with cytokinin. However, the temporal expression of MtNIN in *latd* is different from that in wild type, suggesting the fine-tuning of MtNIN transcriptional regulation is disrupted in *latd*. MtNSP2 is strongly induced by cytokinin treatment in wild type, but this induction is lost in *latd* mutants, providing another evidence of the important role that MtLATD/NIP plays in regulating MtNSP2 expression, both in response to inoculation and to cytokinin. It is interesting that in *latd* mutants, the induction of MtCRE1 and MtRR4 expression by cytokinin is completely wild type, suggesting that cytokinin perception and early signal transduction are intact in *latd*, but not the branch leading to the induction of nodulation specific genes MtNSP2 and MtNIN, both of which are involved in bacterial infection in epidermis and nodule organogenesis in the cortex. It is likely that MtLATD/NIP is an important factor required for both processes via its signaling role in connecting and coordinating these two separate pathways. MtCLE13 expression in *latd* roots is inducible by cytokinin at low levels, but the induction by inoculation is absent in *latd*, suggesting MtLATD/NIP plays a more important role in mediating cytokinin signaling during early nodulation, and that the cytokinin signaling is different in inoculated roots compared to uninoculated root treated with cytokinin. Clearly cell division can happen in the cortex of *latd* mutants after inoculation, since the nodules on *latd* are visible, although they are much smaller in size (Bright et al., 2005). Maybe *latd* mutants are defective in continuously perceiving or maintaining cytokinin signaling, which could explain the observation of an earlier reduction or delayed induction of cytokinin-responsive genes during nodulation. As a nitrate transporter, if transporting nitrate is its only role, then it seems contradictory to the fact that nitrate is a nodulation inhibitor of various developmental stages during nodulation and affects both plant and

bacteria (Stephens and Neyra, 1983; Streeter, 1985, 1985; Malik et al., 1987). Nitrate suppresses early nodulation events such as root hair deformation, IT formation and initial cortical cell division (Malik et al., 1987). Although *latd* mutants can establish these early nodulation events, they do not develop fully. Our results demonstrate that MtLATD/NIP is required for inducing the expression of genes involved in IT development and initiating infection in the epidermis and cortical cell division in the cortex. It is possible that MtLATD/NIP is either transporting another molecule, or transducing another signal that stimulates nodulation.

In summary, we identified 20 TFs that have altered expression levels in *latd* mutant roots, and thus function downstream of MtLATD/NIP. We also identified 79 ABA-regulated TFs in *M. truncatula*, which provides insight to the regulation of ABA during legume root development. The fact that ABA restores the expression of 60% of the mis-expressed TFs in *latd* roots, may provide a mechanism for the rescue by ABA of *latd* root growth defects. MtNSP2 expression is regulated by MtLATD/NIP, ABA and nitrate, and nitrate may achieve this by regulating miR171h expression. MtLATD/NIP also plays a role in mediating cytokinin-responsive gene expression, but only restricted to the genes involved in both cytokinin and Nod factor signaling. *latd* roots failed to exhibit induction of multiple Nod factor signaling genes, which suggests that MtLATD/NIP is required for Nod factor signal transduction and cytokinin crosstalk in nodule organogenesis.

## **MATERIALS AND METHODS**

### **Plant Materials, Growth Conditions and Hormone Treatments**

*M. truncatula* seeds were scarified with concentrated sulfuric acid for 10 minutes, rinsed 6 times with sterile water, and sterilized in 30% Clorox, before imbibing for 5 to 6 hours, shaking, at room temperature. Seeds were cold-treated for at least 24 hours before germinating in a moistened, sealed petri plate overnight in the dark. The A17 line was used as the wild-type control in all experiments. Seedlings were grown on 25 cm x 25 cm petri dishes (Nunc, <http://www.nuncbrand.com/>) or in 15 cm x 15 cm petri dishes (USA Scientific, <http://www.usascientific.com/>), with filter paper over buffered nodulation medium (BNM) at pH 6.5 (Ehrhardt et al., 1992), sealed with Micropore Tape (3M) and placed vertically in an MTR30 Conviron growth chamber at 20°C, 50% humidity, 16 h light/8 h dark cycle with an intensity of 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ . ( $\pm$ )-ABA (A1049; Sigma, <http://www.sigmaaldrich.com/>) or cytokinin (benzyl amino purine) was added to the media after autoclaving to reach specific concentrations. *Sinorhizobium meliloti* strain Rm1021 was grown in liquid Luria broth overnight in the presence of 500  $\mu\text{g/mL}$  streptomycin selection. Bacteria were pelleted at 8,000 rpm for 4 min and resuspended in 10 mM  $\text{MgSO}_4$ . This bacterial suspension was then diluted 1:50 in 10 mM  $\text{MgSO}_4$ , and used for inoculating plants grown on BNM plates by flooding the roots. Extra liquid was then removed and plants were returned to the growth chamber until harvested.

### **TF Expression Profiling**

Total RNA was extracted from whole roots using Trizol reagent, then treated with TURBO DNase Out Kit and followed by Qiagen RNA Clean-up Kit. Each treatment in all experiments was from 20 pooled roots; all experiments were repeated three times. All RNAs were analyzed on an Agilent 2100 Bioanalyzer for quality and integrity. cDNA was synthesized using the SuperScript III first-strand synthesis system (Life Technologies, Invitrogen).



High-throughput qRT-PCR was performed using an ABI 7900HT Real-time PCR system (Applied Biosystems), using SYBR Green reagent (Invitrogen). Data was collected with SDS 2.0 software (Applied Biosystems). PCR efficiency of each individual primer was determined using LinRegPCR software (<http://LinRegPCR.nl>), with data obtained from the exponential phase of each individual amplification. Primers with  $R^2 < 0.95$  and PCR efficiency  $< 0.6$  were excluded from further analysis. Target gene relative expression was determined by normalizing with the geometric mean of the expression of the same eight endogenous controls (Kakar et al., 2008; Gao et al., 2010). Statistical analysis was performed using one-way ANOVA in SPSS software (Version 20.0.0) on the relative gene expression from 3 biological replicates. A p-value  $> 0.05$  and a fold change  $\leq \pm 2$  are considered not significantly different and excluded from further analysis.

#### **qRT-PCR Analysis on Selected TFs**

Total RNA was extracted from whole roots using RNeasy plant mini kits (Qiagen) following the manufacturer's protocol. Each treatment in all experiments was from at least 20 pooled roots; all experiments were repeated at least three times. RNA was quantified with a nanodrop then followed with DNase treatment (Turbo DNase-free kit, Ambion). RNA was subsequently cleaned and concentrated with Qiagen RNA Cleanup Kit. All RNAs were checked with an Agilent 2100 Bioanalyzer for good quality and integrity. cDNA was synthesized from one  $\mu\text{g}$  of total RNA using the SuperScript III first-strand synthesis system (Life Technologies, Invitrogen). qRT-PCR was performed using either an ABI StepOnePlus or ABI 7900HT Real-time PCR system (Applied Biosystems), using SYBR Green reagent (VWR). Data was collected with SDS 2.2 software (Applied Biosystems). Target gene relative expression was determined by normalizing with the expression of endogenous controls, UBC9 and PDF2 (Kakar et al., 2008). Statistical

analysis was performed using one-way ANOVA in SPSS software (Version 20.0.0) on the relative gene expression from 3 biological replicates. Primers used in qRT-PCR were designed using Primer Express v3.0.1 software and are listed in Supplemental Table S6.

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## FIGURE LEGENDS

### **Figure 1. Transcription factors regulated by MtLATD/NIP with and without ABA.**

Transcription factor (TF) families and members of each family that have altered expression levels in *latd* mutant roots without **(A)** and with 10  $\mu$ M ABA **(B)**, compared to wild-type grown under control and with 10  $\mu$ M conditions respectively. TFs shown in the graph have significantly altered gene expression greater than 2-fold, with a p-value <0.05, using one-way ANOVA test. Red text boxes indicate TF families that present in both **(A)** and **(B)**.

### **Figure 2. ABA treatment restores expression of a core group of misregulated TFs in *latd* roots.**

Venn diagrams of transcription factors (TFs) up- **(A)** and down-regulated **(B)** in *latd* mutant roots with (black circle) or without (red circle) the addition of 10  $\mu$ M ABA, as compared to wild-type roots. Only TFs with expression changes greater than 2-fold, and a statistically significant difference between comparisons, with a p-value <0.05, using one-way ANOVA test, are included. **(C)** The heatmap represents relative expression levels of the 20 TFs misregulated in *latd* roots as compared to wild-type (red circles in A and B, first column in C) and the effect of ABA treatment on their expression (black circle in A and B, second column in C). Note that expression of 12/20 TFs misexpressed in *latd* roots is restored to wild-type levels by ABA treatment (black squares in column 2). Relative expression changes and p-values for this heatmap are found in Tables 1 and 2.

### **Figure 3. TF families that are regulated by 10 $\mu$ M ABA in wild-type (A) and *latd* roots (B).**

Graphs display TFs by family, with genes up- and down-regulated by ABA indicated by gray and white bars, respectively. Only TFs whose expression is significantly altered by 10  $\mu$ M ABA more than 2-fold, with a p-value <0.05 in a one-way ANOVA test are included.

**Figure 4. ABA regulates a distinct set of TFs in wild-type and *latd* roots.**

TFs up-regulated (**A**) and down-regulated (**B**) by ABA in wild-type and *latd* roots. Red arrows indicate the percentage corresponding to the number of TFs in the overlapping regions of the Venn diagrams, which represent the ABA-regulated TFs that are shared by both genotypes.

(**C**) The heatmap represents the relative expression of the TFs regulated by ABA in wild-type (blue circles in A and B, first column in C) and *latd* roots (black circles in A and B, second column in C). Only TFs that show expression changes greater than 2-fold, and a statistically significant difference between comparisons, with a p-value <0.05, using a one-way ANOVA test, were included. Overall, only 26/116 TF genes (22.4%) were regulated by ABA in both genotypes.

**Figure 5. The suite of TFs regulated by MtLATD/NIP changes almost completely in the presence of exogenous ABA.**

Numbers of TFs up- (**A**) and down-regulated (**B**) in the *latd* mutant without and with 10  $\mu$ M ABA. Red arrows indicate the percentage of the TFs in the overlapping regions of the Venn diagrams, which are the TFs misregulated in the *latd* mutant in both ABA conditions.

(**C**) The heatmap represents the relative expression of the TFs misregulated in *latd* roots in the absence (green circles in A and B, first column in C) or presence (black circles in A and B, second column in C) of exogenous ABA (10  $\mu$ M ABA). Only TFs that show expression changes greater than 2-fold, and a statistically significant difference between comparisons, with a p-value < 0.05, using a one-way ANOVA test, were included. Overall, only 2/48 TF genes (4.2%) were regulated by ABA in both genotypes.

**Figure 6. MtNSP2 expression in *latd* roots and ABA responses in *nsp2-2* mutants.**

(A) The expression of *MtNSP2* is down-regulated in *latd* mutant roots, and can be repressed further by 10  $\mu$ M ABA in both wild-type (WT) and *latd* roots.

(B-C) Primary root length (B), lateral root number per plant (C) of wild-type (WT) and *nsp2-2* mutants grown continuously on 10  $\mu$ M ABA for 7 days. Graphs in (B) and (C) represent the average of 4 biological replicates, with n=10 for each genotype in each treatment per replicate. Error bars, standard error from four biological replicates. Different letters indicate a statistically significant difference between genotypes and treatment, with a p-value <0.05, using a one-way ANOVA test.

(D) The expression of *RD22*, an ABA-responsive gene, in WT and *nsp2-2* mutant roots grown continuously with and without 10  $\mu$ M ABA for 7 days.

Whole root tissue of 7 day-old plants grown continuously on BNM +/-10  $\mu$ M ABA was harvested and analyzed by qRT-PCR for (A) and (D). Graphs represent the mean +/- standard error from three biological replicates, with n=20 for each genotype in each treatment per replicate. One-way ANOVA was performed to analyze statistical differences (p-value <0.05) for each gene examined. Different letters indicate statistically significant differences between genotypes and treatments. Primers used for qRT-PCR are listed in Supplemental Table S6.

**Figure 7. Relative expression of ABA metabolism-related genes is altered in *nsp2-2* mutant roots.**

Relative expression of two ABA 8'-hydroxylase genes, *Medtr8g076940* (TC165592) (A) and *Medtr1g019410* (TC177719) (B), and an ABA biosynthetic enzyme gene *NCED4* (C) in *nsp2-2* roots with and with 10  $\mu$ M ABA. Plants were grown on BNM agar plates with or without 10  $\mu$ M ABA for 7 days continuously. Graphs represent the average of three biological replicates, with n=15 for each condition per replicate. Different letters indicate statistically significant

differences. One-way ANOVA was performed to analyze statistical differences (p-value <0.05) for each gene examined. Primers are listed in Supplemental Table S6.

**Figure 8. ABA and nitrate regulate the expression of *MtNSP2* and *preMIR171h*.**

Relative expression of *MtNSP2* (A) and *preMIR171h* (B) in wild-type and *latd* mutant roots in response to ABA and nitrate. 7-day old plants were transferred to BNM medium containing 10  $\mu$ M ABA or 10 mM KNO<sub>3</sub> for 3 days. Whole roots were harvested and analyzed for qRT-PCR. Graphs represent the average of three biological replicates, with n=15 for each condition per replicate. Different letters indicate statistically significant difference. One-way ANOVA was performed to analyze statistical differences (p-value <0.05) for each gene examined.

**Figure 9. Root responses of wild-type (WT) and *nsp2-2* mutants to different concentrations of nitrate.**

Plants were grown on BNM medium supplemented with different concentrations of potassium nitrate for 7 days, with corresponding concentrations of potassium chloride as controls. Primary root (A), lateral root density (B) and total lateral root length per cm primary root (C) were measured. Graphs represent the averaged from three biological replicate, n >15 for each genotype per treatment for each replicate. Error bars, standard error of three biological replicates. Asterisks indicate a statistically significant difference between comparisons within each nitrate concentration and control for the same genotype. One-way ANOVA test was performed to analyze statistical differences (p-value <0.05).

**Figure 10. Cytokinin-responsive gene expression in wild-type (WT) and *latd* roots.**

7-day old plants grown on BNM medium were transferred to BNM supplemented with 0.1  $\mu$ M benzyl amino purine (BAP) for 1 hour and 3 hours. Each bar represents an average of three

biological replicates with n=15-20 per replicate. Asterisks represent a statistically significant difference, p-value <0.05.

**(A-F)** Relative expression of *NSP2*, *NIN*, *CLE13*, *CRE1*, *RR1*, and *RR4* in wild-type and *latd* mutant roots in response to 1 hour and 3 hour BAP treatment.

**(G)** Diagram showing branched cytokinin signaling pathway, and MtLATD/NIP is required for the NSP2 branch.

**Figure 11. Relative gene expression in wild-type (WT) and *latd* roots in response to *S. meliloti* inoculation.**

7-day old plants grown on BNM medium were inoculated with *S. meliloti* 1021 and harvested at 1 day post inoculation (dpi) and 2 dpi. Each bar represents an average of three biological replicates with n=15-20 of each genotype per time point per replicate. Asterisks represent a statistically significant difference, p-value <0.05.

**(A-E)** Relative expression of *NSP2*, *NIN*, *CLE13*, *ENOD11*, *ERN1* in wild-type and *latd* mutant roots at 0, 1 and 2 dpi.

**(F)** Diagram showing MtLATD/NIP is required for NSP2-mediated downstream gene induction involved in both bacterial infection in the epidermis and nodule organogenesis in the cortex.

## TABLES

**Table 1. Transcription factors that are down-regulated in *latd* mutant roots.**

Accession Number	Log <sub>2</sub> FC	TF Family	Log <sub>2</sub> FC by ABA
AC136503_46.1	-15.5	CCAAT	0
AC122723_9.2	-2.6	C <sub>2</sub> H <sub>2</sub> (ZF)	0
AC127170_10.2	-2.4	CCHC (ZF)	0
CT009568_4.1	-2.1	C3H (ZF)	0
AC134823_41.1	-1.6	ABI3/VP1	0
AC122723_5.9	-1.5	C <sub>2</sub> H <sub>2</sub> (ZF)	0
CR538722_9.1	-1.4	GRAS (NSP2)	-4.17
AC137839_19.2	-1.2	TTF (ZF)	-1.79
AC149304_11.1	-1.1	bHLH	-1.01

Table 1. Transcription factors down-regulated in *latd* mutant roots. Log<sub>2</sub>FC, Log<sub>2</sub>Fold change of each TF in *latd* roots compared to wild type. Only the TFs that have a Log<sub>2</sub>FC ≤ -1.0 and with a statistically significant difference (p-value < 0.05 in one-way ANOVA test) are listed here. Log<sub>2</sub>FC by ABA is from the comparison of *latd* + 10 μM ABA with wild type at control conditions; red zeros indicate the application of 10 μM ABA restores its expression in *latd* roots to wild-type levels.

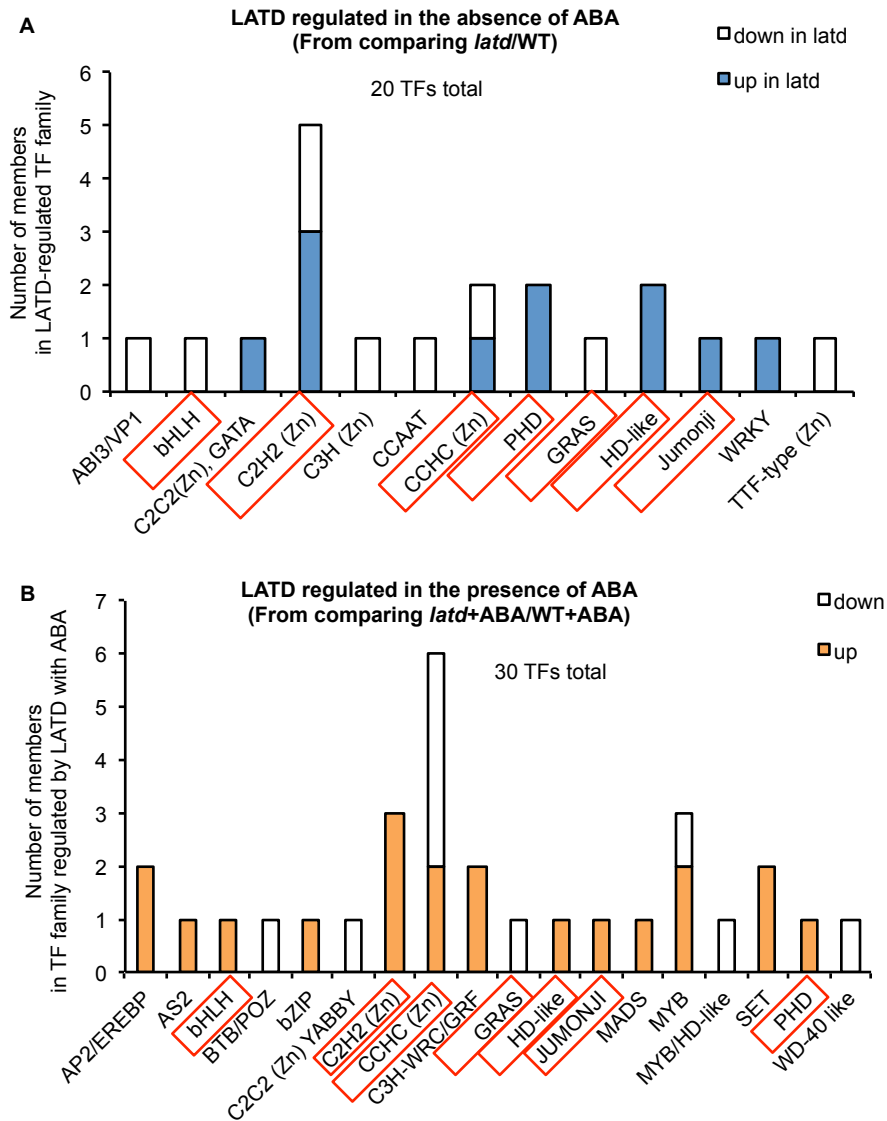


**Table 2. Transcription factor that are up-regulated in *latd* mutant roots.**

Accession No.	Log <sub>2</sub> FC	TF Family	Log <sub>2</sub> FC By ABA
AC151823_11.2	2.2	PHD	0
AC135161_11.7	2.1	WRKY	3.61
AC127019_29.2	1.8	PHD	0
AC130809_24.2	1.7	JUMONJI	2.17
AC152403_40.2	1.5	unknown	1.08
AC124954_14.6	1.3	C <sub>2</sub> H <sub>2</sub> (ZF)	0
AC135959_13.1	1.2	HD-like	5.16
AC135606_17.1	1.1	CCHC (ZF)	0
AC135797_21.1	1.1	CCHC (ZF)	0
AC146329_17.2	1.1	GATA (ZF)	0
AC147009_3.1	1.0	HD-like	0

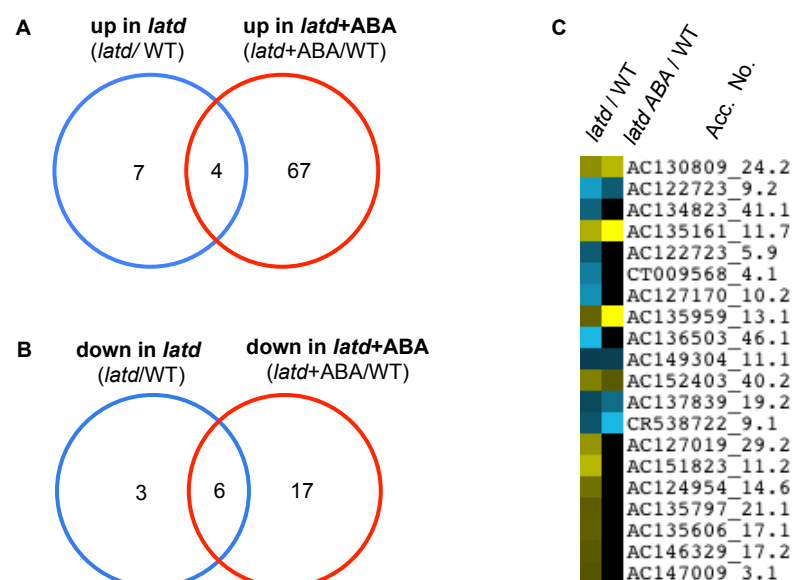
Table 2. Transcription factors up-regulated in *latd* mutant roots. Log<sub>2</sub>FC, Log<sub>2</sub>Fold change of each TF in *latd* roots compared to wild type. Only the TFs that have a Log<sub>2</sub>FC ≥ 1.0 (between *latd* mutants and wild type both at control conditions) and with a statistically significant difference (p-value < 0.05 in one-way ANOVA test) are listed here. Log<sub>2</sub>FC by ABA is from the comparison of *latd* + 10 μM ABA with wild type at control conditions; red zeros indicate the application of 10 μM ABA restores its expression in *latd* roots to wild-type levels.

## FIGURES



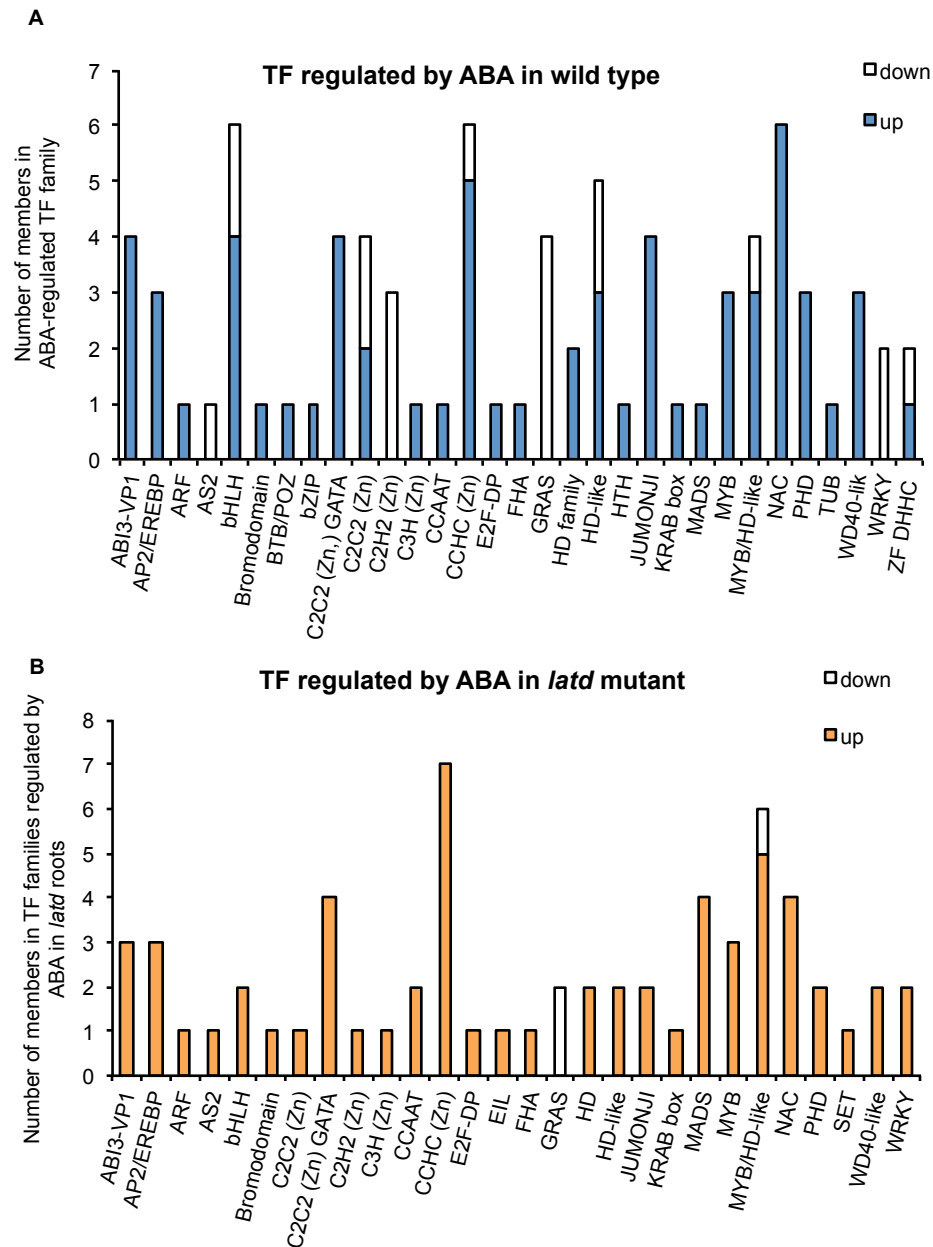
**Figure 1. Transcription factors regulated by MtLATD/NIP with and without ABA.**

Transcription factor (TF) families and members of each family that have altered expression levels in *latd* mutant roots without (A) and with 10  $\mu$ M ABA (B), compared to wild-type grown under control and with 10  $\mu$ M conditions respectively. TFs shown in the graph have significantly altered gene expression greater than 2-fold, with a p-value <0.05, using one-way ANOVA test. Red text boxes indicate TF families that present in both (A) and (B).



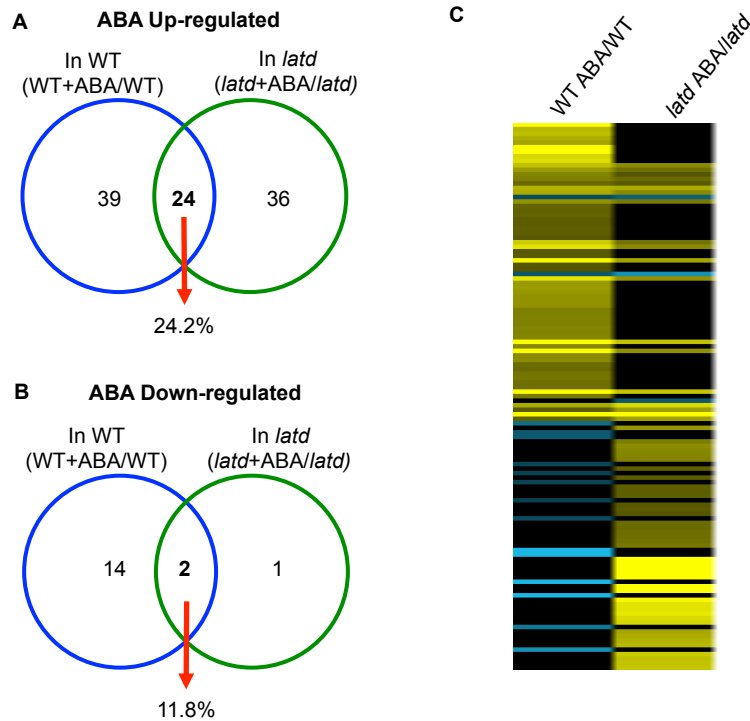
**Figure 2. ABA treatment restores expression of a core group of misregulated TFs in *latd* roots.**

Venn diagrams of transcription factors (TFs) up- (**A**) and down-regulated (**B**) in *latd* mutant roots with (black circle) or without (red circle) the addition of 10  $\mu$ M ABA, as compared to wild-type roots. Only TFs with expression changes greater than 2-fold, and a statistically significant difference between comparisons, with a p-value <0.05, using one-way ANOVA test, are included. (**C**) The heatmap represents relative expression levels of the 20 TFs misregulated in *latd* roots as compared to wild-type (blue circles in A and B, first column in C) and the effect of ABA treatment on their expression (red circle in A and B, second column in C). Note that expression of 12/20 TFs misexpressed in *latd* roots is restored to wild-type levels by ABA treatment (black squares in column 2). Relative expression changes and p-values for this heatmap are found in Tables 1 and 2.



**Figure 3. Transcription factors regulated by ABA in wild-type and *latd* mutant roots.**

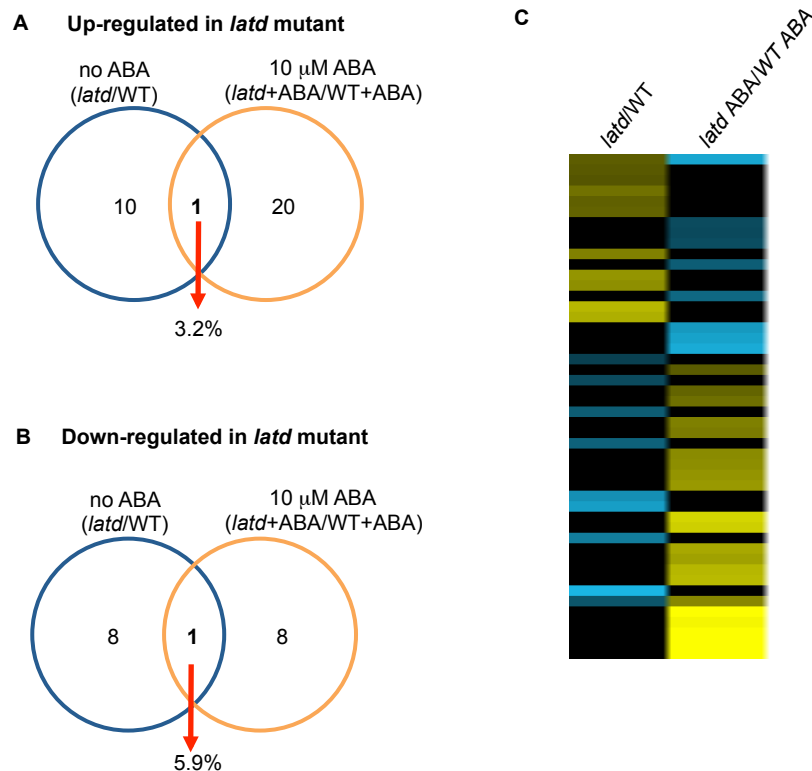
Graphs display TFs by family, with genes up- and down-regulated by ABA indicated by gray and white bars, respectively. Only TFs whose expression is significantly altered by 10  $\mu$ M ABA more than 2-fold, with a p-value <0.05 in a one-way ANOVA test are included.



**Figure 4. ABA regulates a distinct subset of transcription factors in wild-type and *latd* mutant roots.**

TFs up-regulated (A) and down-regulated (B) by ABA in wild-type and *latd* roots. Red arrows indicate the percentage corresponding to the number of TFs in the overlapping regions of the Venn diagrams, which represent the ABA-regulated TFs that are shared by both genotypes.

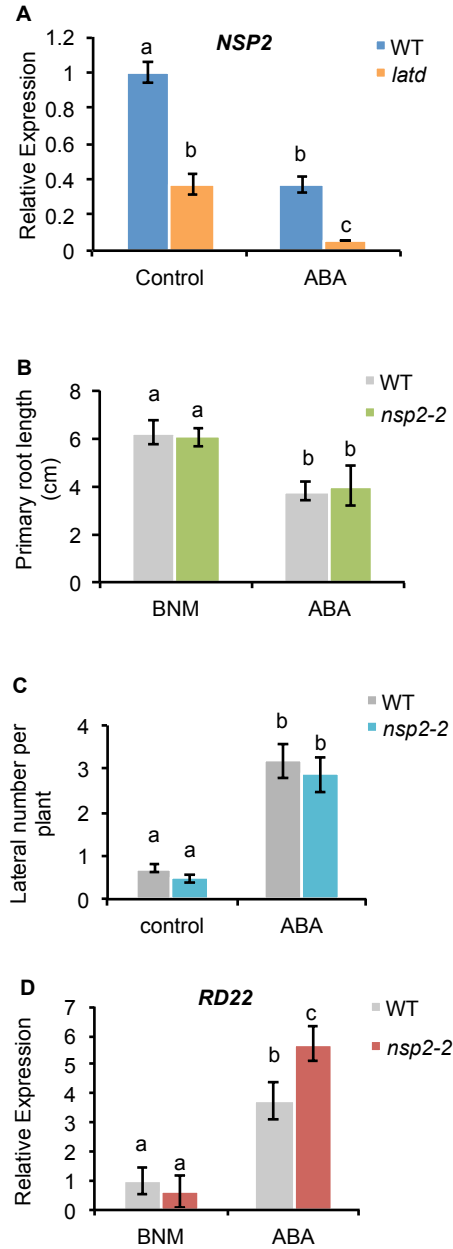
(C) The heatmap represents the relative expression of the TFs regulated by ABA in wild-type (blue circles in A and B, first column in C) and *latd* roots (green circles in A and B, second column in C). Only TFs that show expression changes greater than 2-fold, and a statistically significant difference between comparisons, with a p-value <0.05, using a one-way ANOVA test, were included. Overall, only 26/116 TF genes (22.4%) were regulated by ABA in both genotypes.



**Figure 5. The suite of transcription factors regulated by MtLATD/NIP changes almost completely in the presence of ABA.**

Numbers of TFs up- (A) and down-regulated (B) in the *latd* mutant without and with 10  $\mu$ M ABA. Red arrows indicate the percentage of the TFs in the overlapping regions of the Venn diagrams, which are the TFs misregulated in the *latd* mutant in both ABA conditions.

(C) The heatmap represents the relative expression of the TFs misregulated in *latd* roots in the absence (blue circles in A and B, first column in C) or presence (orange circles in A and B, second column in C) of exogenous ABA (10  $\mu$ M ABA). Only TFs that show expression changes greater than 2-fold, and a statistically significant difference between comparisons, with a p-value  $< 0.05$ , using a one-way ANOVA test, were included. Overall, only 2/48 TF genes (4.2%) were regulated by ABA in both genotypes.



**Figure 6. MtNSP2 expression in *latd* roots and ABA responses in *nsp2-2* mutants.**

**(A)** The expression of MtNSP2 is down-regulated in *latd* mutant roots, and can be repressed further by 10  $\mu$ M ABA in both wild-type (WT) and *latd* roots.

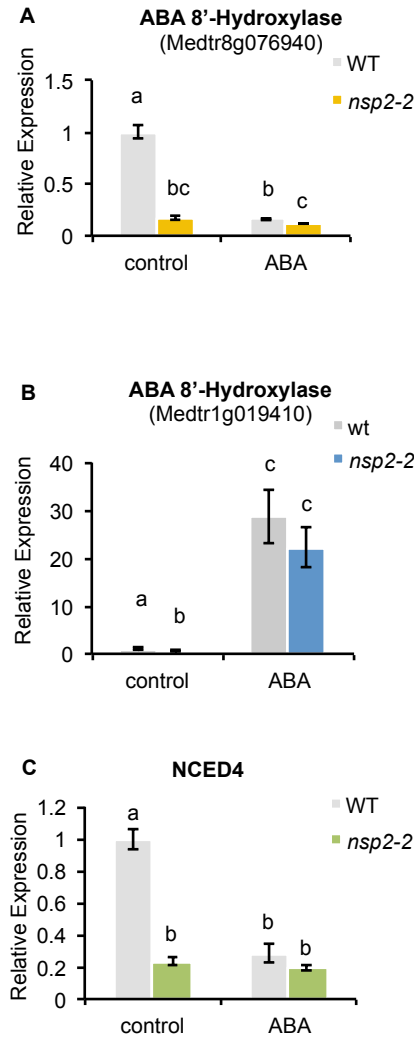
**(B-C)** Primary root length (B), lateral root number per plant (C) of wild-type (WT) and *nsp2-2* mutants grown continuously on 10  $\mu$ M ABA for 7 days. Graphs in (B) and (C) represent the

average of 4 biological replicates, with n=10 for each genotype in each treatment per replicate. Error bars, standard error from four biological replicates. Different letters indicate a statistically significant difference between genotypes and treatment, with a p-value <0.05, using a one-way ANOVA test.

**(D)** The expression of *RD22*, an ABA-responsive gene, in WT and *nsp2-2* mutant roots grown continuously with and without 10  $\mu$ M ABA for 7 days.

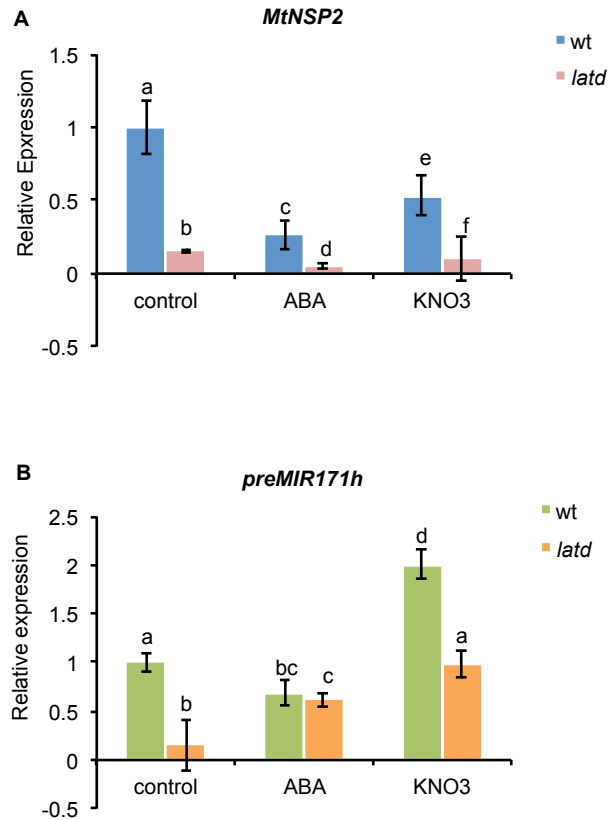
Whole root tissue of 7 day-old plants grown continuously on BNM +/-10  $\mu$ M ABA was harvested and analyzed by qRT-PCR for (A) and (D). Graphs represent the mean +/- standard error from three biological replicates, with n=20 for each genotype in each treatment per replicate. One-way ANOVA was performed to analyze statistical differences (p-value <0.05) for each gene examined. Different letters indicate statistically significant differences between genotypes and treatments. Primers used for qRT-PCR are listed in Supplemental Table S6.





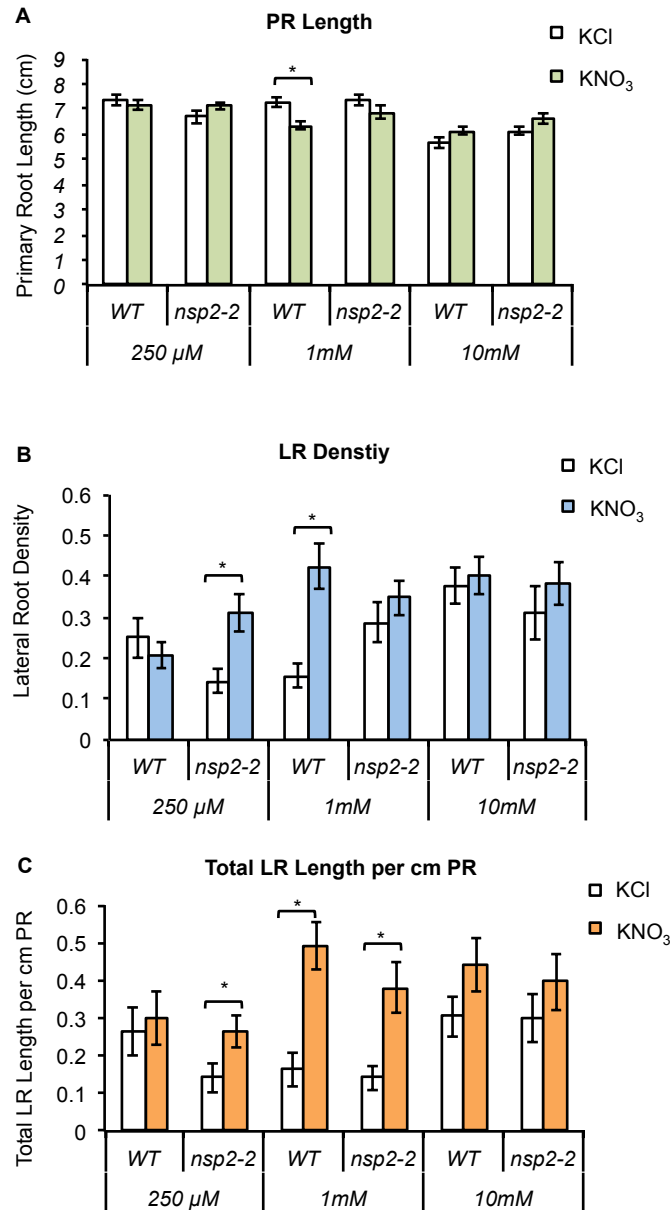
**Figure 7. Relative expression of ABA metabolism-related genes is altered in *nsp2-2* mutant roots.**

Relative expression of two ABA 8'-hydroxylase genes, Medtr8g076940 (TC165592) (**A**) and Medtr1g019410 (TC177719) (**B**), and an ABA biosynthetic enzyme gene *NCED4* (**C**) in *nsp2-2* roots with and with 10  $\mu$ M ABA. Plants were grown on BNM agar plates with or without 10  $\mu$ M ABA for 7 days continuously. Graphs represent the average of three biological replicates, with  $n=15$  for each condition per replicate. Different letters indicate statistically significant differences. One-way ANOVA was performed to analyze statistical differences ( $p$ -value  $<0.05$ ) for each gene examined. Primers are listed in Supplemental Table S6.



**Figure 8. ABA and nitrate regulate the expression of *MtNSP2* and *preMIR171h*.**

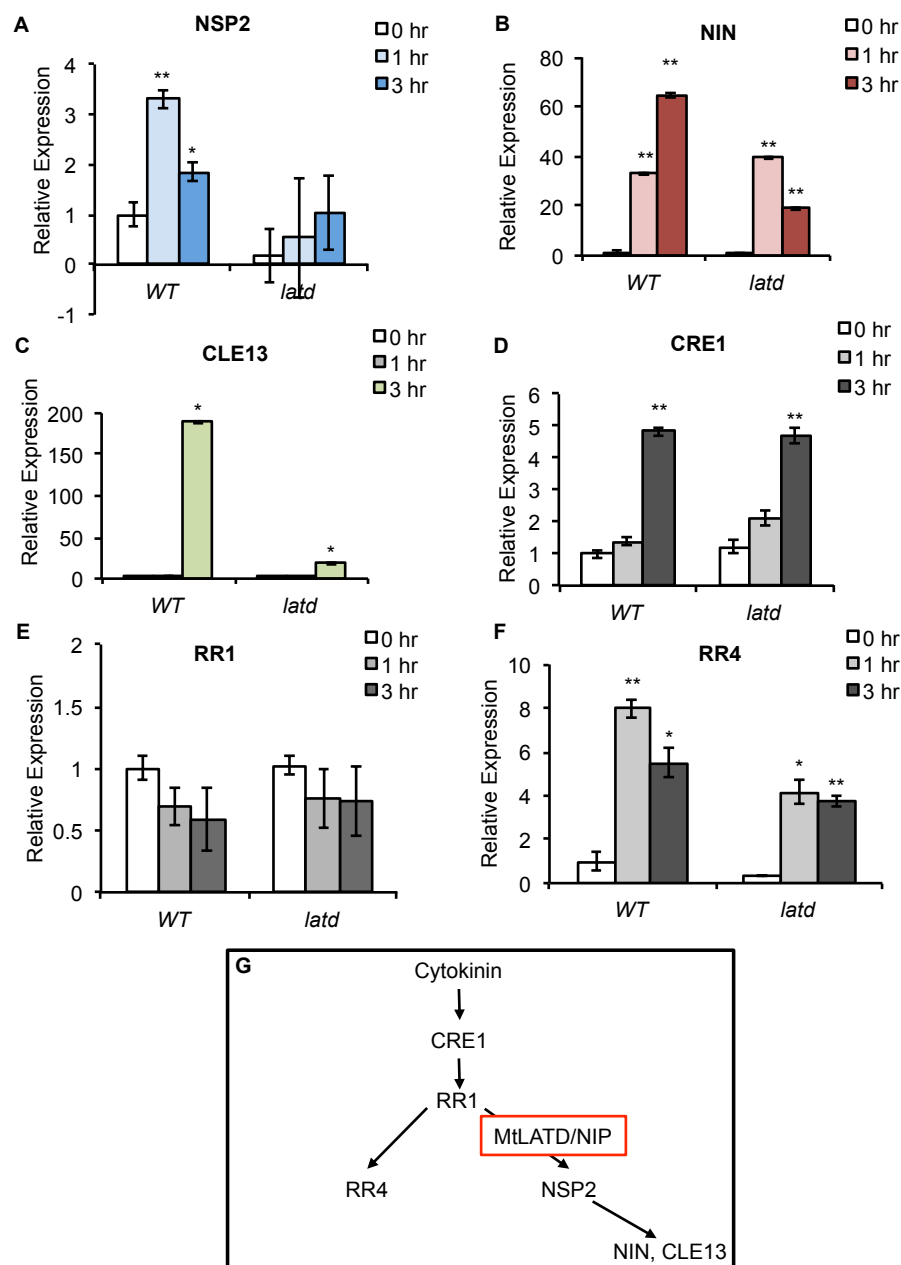
Relative expression of *MtNSP2* (**A**) and *preMIR171h* (**B**) in wild-type and *latd* mutant roots in response to ABA and nitrate. 7-day old plants were transferred to BNM medium containing 10  $\mu$ M ABA or 10 mM KNO<sub>3</sub> for 3 days. Whole roots were harvested and analyzed for qRT-PCR. Graphs represent the average of three biological replicates, with n=15 for each condition per replicate. Different letters indicate statistically significant difference. One-way ANOVA was performed to analyze statistical differences (p-value <0.05) for each gene examined.



**Figure 9. Root responses to nitrate in *nsp2-2* mutant roots.**

Plants were grown on BNM medium supplemented with different concentrations of potassium nitrate for 7 days, with corresponding concentrations of potassium chloride as controls. Primary root (A), lateral root density (B) and total lateral root length per cm primary root (C) were measured. Graphs represent the averaged from three biological replicate,  $n > 15$  for each genotype per treatment for each replicate. Error bars, standard error of three biological replicates. Asterisks

indicate a statistically significant difference between comparisons within each nitrate concentration and control for the same genotype. One-way ANOVA test was performed to analyze statistical differences (p-value <0.05).

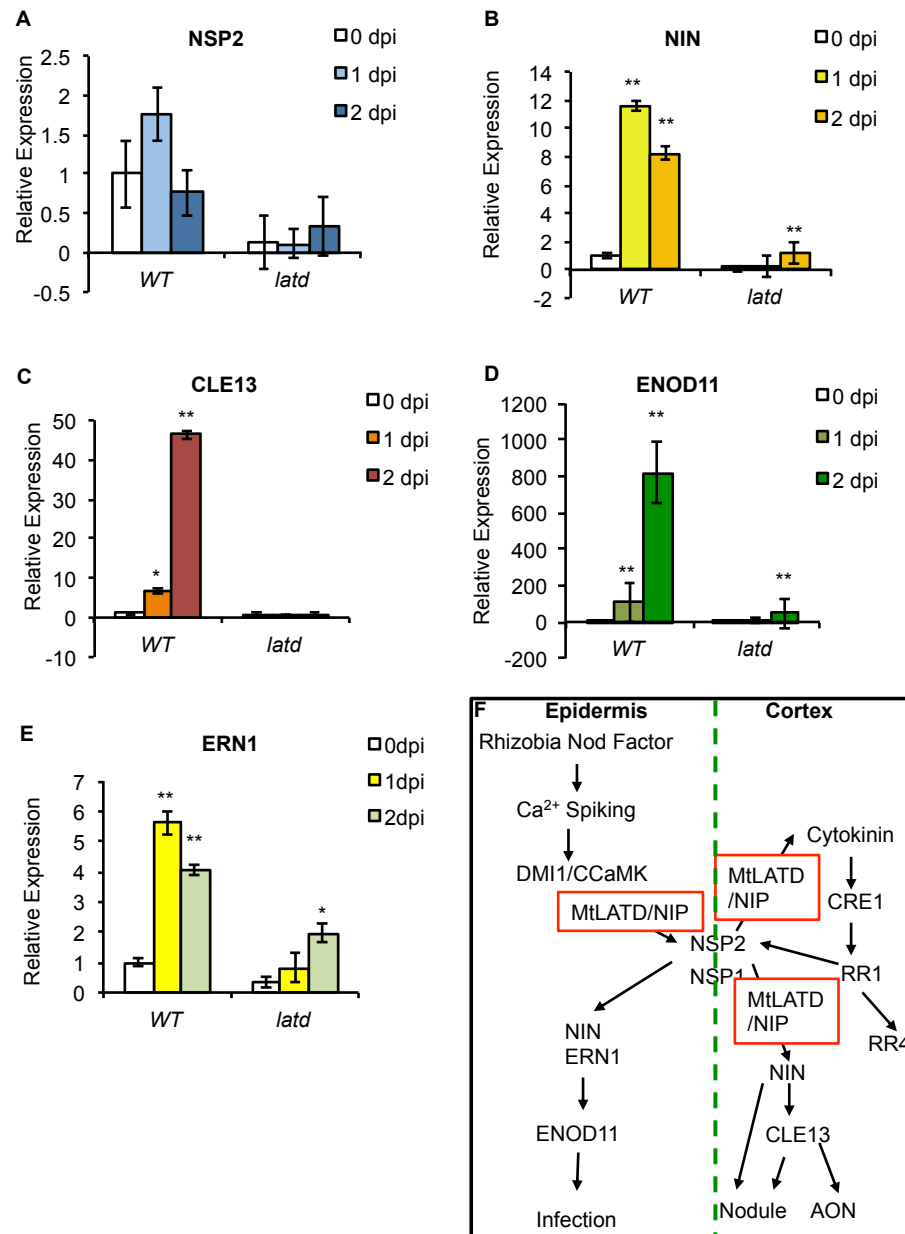


**Figure 10. Cytokinin-responsive gene expression in wild-type and *latd* mutant roots.**

7-day old wild-type (WT) and *latd* plants grown on BNM medium were transferred to BNM supplemented with 0.1  $\mu$ M benzyl amino purine (BAP) for 1 hour and 3 hours. Each bar represents an average of three biological replicates with n=15-20 per replicate. Asterisks represent a statistically significant difference, p-value <0.05.

**(A-F)** Relative expression of *NSP2*, *NIN*, *CLE13*, *CRE1*, *RR1*, and *RR4* in wild-type and *latd* mutant roots in response to 1 hour and 3 hour BAP treatment.

**(G)** Diagram showing branched cytokinin signaling pathway, and MtLATD/NIP is required for the NSP2 branch.



**Figure 11. Relative gene expression in wild-type (WT) and *latd* mutant roots in response to *S. meliloti* inoculation.**

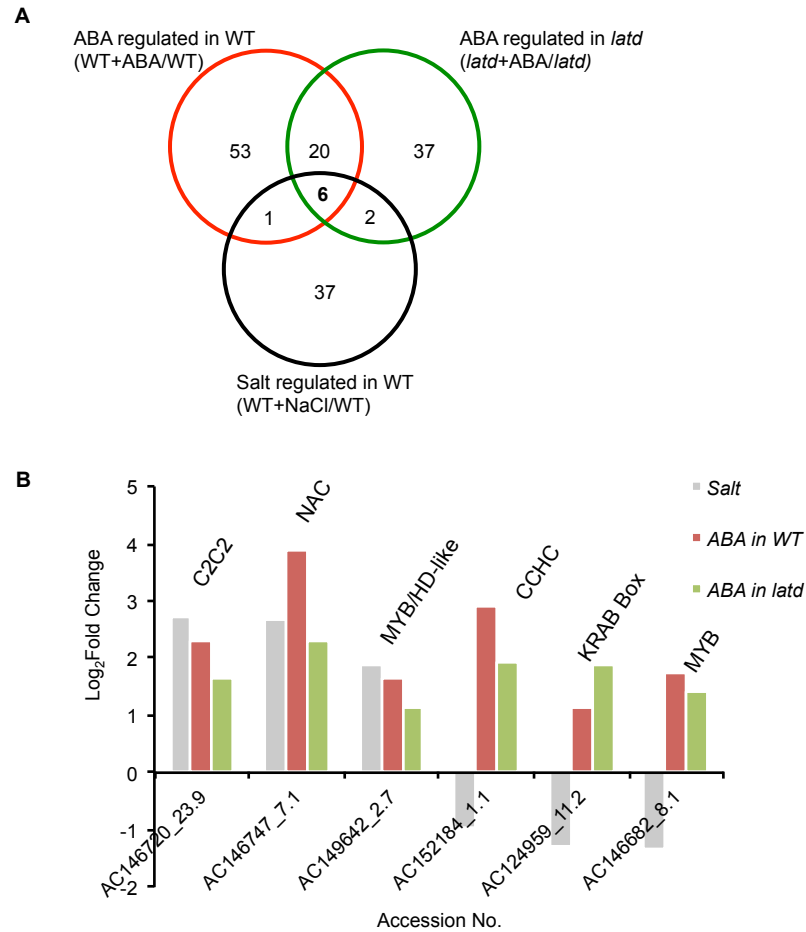
7-day old plants grown on BNM medium were inoculated with *S. meliloti* 1021 and harvested at 1 day post inoculation (dpi) and 2 dpi. Each bar represents an average of three biological replicates with n=15-20 of each genotype per time point per replicate. Asterisks represent a statistically significant difference, p-value <0.05.

**(A-E)** Relative expression of *NSP2*, *NIN*, *CLE13*, *ENOD11*, *ERN1* in wild-type and *latd* mutant roots at 0, 1 and 2 dpi.

**(F)** Diagram showing MtLATD/NIP is required for NSP2-mediated downstream gene induction involved in both bacterial infection in the epidermis and nodule organogenesis in the cortex.



## SUPPLEMENTAL MATERIALS



**Supplemental Figure S1.** TFs regulated by ABA and salt stress.

TFs regulated by high salt are from Gruber et al., where two-week-old plants were agitated in liquid growth media containing 100 mM NaCl for 1 hour before harvested. ABA-regulated TFs are from this study, where 4 day-old plants grown continuously on agar plates with growth medium BNM with 10  $\mu$ M ABA.

(A) Venn diagram of the number of TFs regulated by salt (in wild-type) and by ABA (in wild type and *latd* mutants). Six TFs are regulated by salt, and by ABA in both wild-type and *latd* mutant roots.

(B) Log<sub>2</sub>Fold changes (Log<sub>2</sub>FC) of TFs regulated by salt stress (in wild type) and by ABA (in wild type and *latd* mutant), which are in the overlapping regions of venn diagrams shown in (A). The TF family of each TF is shown on top of the bars in the graph.

**Supplemental Table S1.** TFs that have altered expression levels in *latd* roots treated with 10  $\mu$ M, compared to wild type.

Accession no.	log <sub>2</sub> FC ( <i>latd</i> ABA/WT)	p- value	TF family	Gene Expression Atlas
CT009492_35.1	1.3896	0.004	ABI3-VP1	Mtr.24484.1.S1_at
AC119408_11.1	-1.0828	0.017	AP2/EREBP	N
AC137546_7.1	1.6868	0.002	AP2/EREBP	N
AC126784_13.2	2.3331	0.004	AP2/EREBP	N
AC148348_6.1	3.1875	0.03	AP2/EREBP	Mtr.20228.1.S1_at
AC144645_17.6	3.9286	0.036	AP2/EREBP	Mtr.18880.1.S1_at
AC137994_19.3	5.3067	0.02	AP2/EREBP	Mtr.46362.1.S1_at
AC148995_2.1	1.9277	0.041	ARF	N
AC148995_2.8	3.8804	0.041	ARF	N
AC146329_17.1	2.9202	0.033	ARID	Mtr.19628.1.S1_at
AC144729_15.1	-1.7514	0.009	AS2	N
CR378662_19.1	1.6965	0.006	AS2	N
AC155896_2.1	2.8594	0.043	AS2	N
AC149268_1.2	3.1933	0.001	AS2	N
AC125478_7.2	-1.0168	0.044	bHLH	N
AC123898_40.1	1.2736	0.042	bHLH	Mtr.14692.1.S1_at
AC137831_27.1	2.2410	0.002	bHLH	Mtr.652.1.S1_at
AC148528_15.1	3.6855	0.016	bHLH	N
AC148528_15.1	5.2834	0.01	bHLH	N
AC125478_13.7	-2.8329	0.018	bZIP	Mtr.15416.1.S1_at
AC140545_26.2	1.3037	0.016	bZIP	Mtr.19224.1.S1_at
AC125481_22.1	3.2794	0.028	bZIP	Mtr.49089.1.S1_at
AC152403_40.2	1.0782	0.016	C2C2 (Zn)	N
AC126019_15.2	1.1684	0.049	C2C2 (Zn)	N
			Dof	
			C2C2 (Zn)	
AC147405_7.6	-2.2002	0.046	YABBY	Mtr.21678.1.S1_at
AC122723_9.2	-1.5257	0.027	C2H2 (Zn)	N
AC161241_23.1	-1.1761	0.017	C2H2 (Zn)	Mtr.23454.1.S1_at
AC135797_21.1	1.3196	0.028	C2H2 (Zn)	N
AC148340_39.2	1.1984	0.025	C3H (Zn)	N
CT010521_14.1	-1.2718	0.026	CCAAT	Mtr.40976.1.S1_at
AC136138_30.1	1.6171	0.003	CCAAT	Mtr.51511.1.S1_at
AC146308_2.1	1.8436	0.049	CCAAT	Mtr.51561.1.S1_at
AC148226_7.1	-7.1093	0.026	CCHC (Zn)	Mtr.49901.1.S1_at
AC144807_23.9	1.5970	0.042	CCHC (Zn)	Mtr.19164.1.S1_at
AC149264_8.1	1.8661	0.019	CCHC (Zn)	Mtr.21213.1.S1_at

AC152184_1.1	2.6137	0.026	CCHC (Zn)	N
Accession no.	log <sub>2</sub> FC ( <i>latd</i> ABA/WT)	p- value	TF family	Gene Expression Atlas
AC138056_33.2	4.1591	0.033	CCHC (Zn)	Mtr.17993.1.S1_at
AC121233_16.1	12.1280	0.016	CCHC (Zn)	Mtr.49200.1.S1_at
AC137065_19.2	1.9442	0.029	E2F-DP	N
AC152185_34.1	-5.0449	0.002	GRAS	N
CR538722_9.1	-4.1658	0	GRAS	Mtr.44789.1.S1_at
AC152185_33.1	-1.7256	0.001	GRAS	N
AC147430_9.6	2.2746	0.008	GRF	Mtr.21717.1.S1_x_a t
AC157472_19.1	-2.6934	0.02	HD	N
CT009553_7.1	-1.1426	0.003	HD	Mtr.6737.1.S1_s_at
AC137078_24.8	1.1160	0.016	HD	Mtr.51678.1.S1_at
AC146571_13.2	2.1495	0.042	HD-like	Mtr.20096.1.S1_at
AC146788_13.1	5.1624	0.03	HD-like	N
AC144592_24.3	1.5075	0.032	HD-ZIP	Mtr.18769.1.S1_at
AC144592_26.4	1.5378	0.028	HD-ZIP	N
AC144759_11.1	1.8486	0.038	JUMONJI	Mtr.19107.1.S1_at
AC130809_36.1	1.9460	0.025	JUMONJI	N
AC130809_24.2	2.1658	0.033	JUMONJI	Mtr.51283.1.S1_at
AC144644_3.9	1.8571	0.039	MADS	Mtr.18866.1.S1_at
AC122160_21.1	2.0852	0.049	MADS	Mtr.52263.1.S1_at
AC144731_15.2	2.3786	0.021	MADS	Mtr.19093.1.S1_at
AC144517_7.2	2.5178	0.016	MADS	Mtr.50752.1.S1_at
AC148527_19.1	3.0946	0.027	MADS	N
AC136140_17.1	-2.9502	0.027	MYB	Mtr.16873.1.S1_s_at
AC147536_10.1	-2.7441	0.016	MYB	N
AC147712_17.2	-2.6017	0	MYB	N
AC129092_13.1	-2.2087	0.028	MYB	Mtr.16432.1.S1_at
AC157502_5.1	1.4920	0.003	MYB	N
AC146817_10.1	2.1750	0.032	MYB	Mtr.21143.1.S1_at
AC149129_3.1	2.7148	0.017	MYB	Mtr.31535.1.S1_at
AC139600_7.2	2.7838	0.008	MYB	N
AC149642_7.1	3.5665	0.001	MYB	N
AC144807_23.4	7.3598	0.032	MYB	Mtr.50305.1.S1_at
AC147499_9.2	1.3687	0.016	MYB/HD- like	N
AC123899_49.2	3.2996	0.016	MYB/HD- like	N

AC144807_5.2	7.4191	0.03	MYB/HD-like	N
AC140544_19.1	1.3844	0.026	NAC	Mtr.19055.1.S1_at
AC122164_5.1	1.6291	0.027	NAC	Mtr.15417.1.S1_at
Accession no.	$\log_2FC$ ( <i>latd</i> ABA/WT)	p-value	TF family	Gene Expression Atlas
AC140030_6.1	2.0488	0.016	NAC	Mtr.19019.1.S1_at
AC145753_1.1	2.5497	0.024	NAC	Mtr.50038.1.S1_at
AC150891_8.1	4.1693	0	NAC	N
AC126007_16.1	1.5138	0.029	PHD	Mtr.50981.1.S1_at
AC130809_24.8	2.7035	0.026	PHD	Mtr.51289.1.S1_s_at
AC144541_12.8	3.6189	0.041	PHD	Mtr.18668.1.S1_at
CR932967_9.2	4.1208	0.029	PHD	N
AC146785_2.1	-1.3383	0.001	RR	N
AC139601_8.1	1.8746	0.043	SNF2	Mtr.18515.1.S1_at
AC146745_18.2	1.5963	0.016	SRS	Mtr.45803.1.S1_at
AC144502_5.3	-1.6787	0.044	TF	Mtr.18512.1.S1_at
AC137839_19.2	-1.7928	0.017	TTF-type (Zn)	N
AC146806_13.3	1.3360	0.037	TTF-type (Zn)	Mtr.46223.1.S1_s_at
MTR_7g086050	1.2171	0.048	unknown	N
AC144617_10.1	1.0295	0.025	WD40-like	Mtr.18818.1.S1_at
AC149491_2.7	3.1741	0.039	WD40-like	Mtr.21379.1.S1_at
AC135465_23.2	1.0188	0.007	WRKY	N
AC135465_24.1	1.5320	0.001	WRKY	N
AC135161_11.7	3.6094	0.016	WRKY	Mtr.52359.1.S1_at
AC126786_22.1	-10.4656	0.022	ZF DHHC	Mtr.15927.1.S1_at

**Supplemental Table S2.** TFs whose expression levels are significantly altered by ABA in wild-type *M. truncatula* root.

Accession no.	Log <sub>2</sub> FC (WT+ABA/WT)	p-value	TF Family	Gene Expression Atlas
AC148404_7.9	1.0772	0.0407	ABI3-VP1	Mtr.20288.1.S1_at
AC146747_7.151	1.2799	0.0458	ABI3-VP1	Mtr.20853.1.S1_at
AC148404_7.7	2.1989	0.0110	ABI3-VP1	Mtr.49505.1.S1_at
AC148404_7.2	3.2824	0.0120	ABI3-VP1	Mtr.20280.1.S1_at
AC143341_8.181	1.1532	0.0477	AP2/EREBP	Mtr.18059.1.S1_at
AC145767_15.2	1.1993	0.0432	AP2/EREBP	Mtr.19570.1.S1_at
AC148348_6.181	1.5059	0.0328	AP2/EREBP	Mtr.20228.1.S1_at
AC148995_2.101	1.0434	0.0014	ARF	N
AC144729_15.1	-1.4764	0.0467	AS2	N
AC135231_23.101	-1.1618	0.0428	bHLH	Mtr.45719.1.S1_at
AC149304_11.161	-1.0911	0.0132	bHLH	Mtr.35725.1.S1_at
AC141114_14.1	1.1046	0.0267	bHLH	Mtr.14223.1.S1_at
AC149304_11.181	1.3779	0.0153	bHLH	Mtr.7448.1.S1_s_at
AC146865_11.1	1.8150	0.0020	bHLH	Mtr.21313.1.S1_at
AC146548_9.5	2.2732	0.0230	bHLH	Mtr.19721.1.S1_at
AC144564_12.2	1.4954	0.0030	Bromodomain	Mtr.37707.1.S1_at
AC154867_1.2	1.1234	0.0268	n	Mtr.1948.1.S1_at
AC144805_7.1	1.7311	0.0130	BTB/POZ	Mtr.50324.1.S1_a_at
AC146745_18.1	2.3982	0.0450	bZIP	Mtr.20821.1.S1_at
AC147428_6.2	1.2449	0.0263	C <sub>2</sub> C <sub>2</sub> (Zn) C <sub>2</sub> C <sub>2</sub> (Zn), GATA	N
AC147428_6.6	1.8317	0.0030	C <sub>2</sub> C <sub>2</sub> (Zn), GATA	Mtr.48513.1.S1_at
AC146329_57.2	2.0152	0.0010	C <sub>2</sub> C <sub>2</sub> (Zn), GATA	N
AC146720_23.9	2.2780	0.0410	C <sub>2</sub> C <sub>2</sub> (Zn), GATA	Mtr.20716.1.S1_at
AC122723_9.2	-3.1467	0.0079	C <sub>2</sub> H <sub>2</sub> (Zn)	N
AC122723_5.9	-3.0504	0.0307	C <sub>2</sub> H <sub>2</sub> (Zn)	Mtr.49394.1.S1_at
AC139344_17.2	1.6255	0.0450	C <sub>2</sub> H <sub>2</sub> (Zn)	Mtr.18387.1.S1_at
AC148340_39.2	2.5435	0.0090	C3H	N
AC136138_30.1	1.5228	0.0111	CCAAT	Mtr.51511.1.S1_at
AC146683_9.1	-1.1297	0.0092	CCHC (Zn)	N
AC149264_8.1	1.0080	0.0488	CCHC (Zn)	Mtr.21213.1.S1_at
AC152184_1.1	2.9210	0.0210	CCHC (Zn)	N

AC144538_22.6	10.2823	0.0404	CCHC (Zn)	N
<b>Accession no.</b>	<b>Log<sub>2</sub>FC</b>	<b>p-value</b>	<b>TF Family</b>	<b>Gene Expression Atlas</b>
AC121233_16.1	12.2568	0.0054	CCHC (Zn)	Mtr.49200.1.S1_at
AC146777_27.1	1.7341	0.0170	CCHC (Zn)	Mtr.20961.1.S1_at
AC137065_19.2	1.5594	0.0430	E2F-DP	N
AC126782_22.2	2.0394	0.0020	FHA	Mtr.15843.1.S1_at
AC152185_34.1	-3.1829	0.0180	GRAS	N
AC152185_34.2	-1.9929	0.0356	GRAS	N
CR538722_9.1	-1.4282	0.0405	GRAS	Mtr.44789.1.S1_at
AC152185_1.321	-1.2482	0.0104	GRAS	N
AC146692_14.4	1.0309	0.0416	HD	Mtr.20585.1.S1_at
AC144724_8.3	2.0645	0.0080	HD	Mtr.18980.1.S1_at
AC127170_10.2	-2.3682	0.0172	HD-like	N
AC146571_13.2	-1.7855	0.0363	HD-like	Mtr.20095.1.S1_at
AC147009_3.1	1.1101	0.0469	HD-like	N
AC146571_13.261	1.5875	0.0180	HD-like	Mtr.20096.1.S1_at
AC148994_3.9	1.7800	0.0480	HD-like	Mtr.20801.1.S1_at
AC124218_17.7	1.0711	0.0190	HTH	Mtr.14768.1.S1_at
AC166147_22.2	1.1179	0.0440	JUMONJI	N
AC149642_2.1	1.3892	0.0020	JUMONJI	Mtr.21459.1.S1_at
AC151823_11.2	1.5339	0.0160	JUMONJI	N
AC144759_11.1	2.1255	0.0050	JUMONJI	Mtr.19107.1.S1_at
AC124959_11.2	1.1459	0.0052	KRAB box	Mtr.52293.1.S1_at
AC144644_3.9	1.8434	0.0240	MADS	Mtr.18866.1.S1_at
AC146682_8.1	1.7260	0.0040	MYB	N
AC147536_10.141	2.6255	0.0040	MYB	N
AC146817_10.1	2.6865	0.0060	MYB	Mtr.21143.1.S1_at
AC139707_10.7	-1.2699	0.0415	MYB/HD-like	Mtr.18579.1.S1_at
AC124972_40.131	1.0033	0.0309	MYB/HD-like	Mtr.52042.1.S1_s_at
AC149642_2.7	1.6468	0.0220	MYB/HD-like	Mtr.21051.1.S1_at
AC146760_13.7	1.9414	0.0020	MYB/HD-like	Mtr.20924.1.S1_at
AC126789_18.4	1.1660	0.0008	NAC	Mtr.51152.1.S1_at
AC140544_19_1	1.2585	0.0262	NAC	Mtr.50401.1.S1_at
AC122164_5.1	1.5490	0.0200	NAC	Mtr.15417.1.S1_at
AC140544_19.1	1.5618	0.0493	NAC	Mtr.19055.1.S1_at
AC145753_1.1	2.6945	0.0050	NAC	Mtr.50038.1.S1_at
AC146747_7.1	3.9008	0.0050	NAC	Mtr.20852.1.S1_at

AC126007_16.171	1.3120	0.0229	PHD	Mtr.50981.1.S1_at
AC155099_1.2	1.7205	0.0100	PHD	N
AC144541_12.8	3.6494	0.0080	PHD	Mtr.18668.1.S1_at
<b>Accession no.</b>	<b>Log<sub>2</sub>FC</b>	<b>p-value</b>	<b>TF Family</b>	<b>Gene Expression Atlas</b>
AC151598_2.411	1.3149	0.0397	TUB	N
AC149801_3.1	3.1998	0.0120	WD-40 like	Mtr.21482.1.S1_at
AC144617_10.1	1.6314	0.0318	WD40-like	Mtr.18818.1.S1_at
AC149491_2.7	3.3343	0.0170	WD40-like	Mtr.21379.1.S1_at
AC133571_10.1	-1.4915	0.0426	WRKY	N
AC122726_21.111	-1.0527	0.0488	WRKY	Mtr.15568.1.S1_s
AC126786_22.1	-10.3213	0.0372	ZF DHHC	_at
AC146747_48.2	1.4346	0.0210	ZF DHHC	Mtr.15927.1.S1_at
AC123899_15.181	1.5313	0.0490	Zn finger, LIM	Mtr.20850.1.S1_at
				Mtr.52015.1.S1_at



**Supplemental Table S3.** TFs whose expression levels are significantly altered by ABA in *latd* mutant root.

Accession no.	Log <sub>2</sub> FC ( <i>latd</i> +ABA / <i>latd</i> )	p-value	TF family	Gene Expression Atlas
AC146747_7.1	1.6684	0.013	ABI3-VP1	Mtr.20853.1.S1_at
AC148404_7.2	2.7028	0.002	ABI3-VP1	Mtr.20280.1.S1_at
AC148404_7.7	1.8703	0.002	ABI3-VP1	Mtr.49505.1.S1_at
AC144645_17.6	2.6753	0.028	AP2/EREBP	Mtr.18880.1.S1_at
AC148348_6.1	2.1222	0.036	AP2/EREBP	Mtr.20228.1.S1_at
AC137994_19.3	2.7522	0.023	AP2/EREBP	Mtr.46362.1.S1_at
AC148995_2.8	3.7668	0.027	ARF	N
AC140849_4.1	2.9288	0.042	AS2	Mtr.17172.1.S1_at
AC148528_15.1	4.1065	0.039	bHLH	N
AC140671_10.4	1.2719	0.030	bHLH	Mtr.50163.1.S1_at
AC144564_12.2	1.2631	0.054	Bromodomain	Mtr.50680.1.S1_at
AC146745_18.1	1.3538	0.000	n	Mtr.20821.1.S1_at
AC147428_6.2	1.0781	0.020	C <sub>2</sub> C <sub>2</sub> (Zn)	Mtr.20821.1.S1_at
AC147428_6.6	1.3238	0.006	C <sub>2</sub> C <sub>2</sub> (Zn)	N
AC146329_57.2	2.5859	0.000	GATA	N
AC146720_23.9	1.6493	0.012	C <sub>2</sub> C <sub>2</sub> (Zn)	Mtr.48513.1.S1_at
AC128638_34.1	1.0695	0.009	GATA	N
AC152184_1.1	1.9240	0.006	C <sub>2</sub> C <sub>2</sub> (Zn)	Mtr.20716.1.S1_at
AC203547_3.1	1.1098	0.042	GATA	Mtr.49025.1.S1_at
AC121233_16.1	13.0845	0.000	C <sub>2</sub> H <sub>2</sub> (Zn)	N
AC138056_33.2	2.5806	0.031	CCHC (Zn)	N
AC140549_11.2	1.0953	0.000	CCHC (Zn)	Mtr.49200.1.S1_at
AC123570_7.1	1.0481	0.035	CCHC (Zn)	Mtr.17993.1.S1_at
AC150777_2.1	2.2757	0.027	CCHC (Zn)	Mtr.19386.1.S1_at
AC137837_3.3	2.1762	0.002	CCHC (Zn)	N
AC146308_2.1	1.6039	0.003	CCHC (Zn)	N
AC136503_46.1	16.0093	0.000	CCAAT	N
AC137065_19.2	1.2977	0.017	CCAAT	N
AC131455_9.1	1.3122	0.020	E2F-DP	N
AC126782_22.2	1.6394	0.017	EIL	N
CR538722_9.1	-2.3951	0.025	FHA	Mtr.15843.1.S1_at
			GRAS	Mtr.44789.1.S1_at

AC152185_1.321	-1.5851	0.040	GRAS	N
AC144724_8.3	1.3451	0.007	HD	Mtr.18980.1.S1_at
Accession no.	Log <sub>2</sub> FC	p-value	TF family	Gene Expression Atlas
AC139525_3.5	1.4920	0.022	HD	Mtr.18411.1.S1_s_at
AC144766_18.5	3.4254	0.001	HD-like	N
AC119415_25.1	7.1446	0.002	HD-like	N
AC144759_11.1	1.2675	0.012	JUMONJI	Mtr.19107.1.S1_at
AC141323_7.1	2.0306	0.007	JUMONJI	Mtr.14445.1.S1_at
AC124959_11.2	1.8800	0.003	KRAB box	Mtr.52293.1.S1_at
AC161107_33.2	1.5854	0.009	MADS	N
AC148527_19.1	2.3178	0.018	MADS	N
AC144731_15.2	1.9693	0.031	MADS	Mtr.19093.1.S1_at
AC146650_13.3	4.1660	0.049	MADS	Mtr.20461.1.S1_s_at
AC146682_8.1	1.4271	0.021	MYB	N
AC146817_10.1	1.6345	0.005	MYB	Mtr.21143.1.S1_at
AC157502_5.1	2.3903	0.009	MYB	N
AC147499_9.2	1.8018	0.015	MYB/HD-like	N
AC123899_49.2	2.3426	0.015	MYB/HD-like	N
AC123899_15.1	1.0150	0.023	MYB/HD-like	Mtr.51999.1.S1_at
AC149642_2.7	1.1446	0.006	MYB/HD-like	Mtr.21051.1.S1_at
AC149129_3.1	3.2399	0.001	MYB/HD-like	Mtr.31535.1.S1_at
AC152184_1.2	-1.4628	0.032	like	N
AC146747_7.1	2.2951	0.002	NAC	Mtr.20852.1.S1_at
AC145753_1.1	1.7343	0.007	NAC	Mtr.50038.1.S1_at
AC140544_19_1	1.0196	0.010	NAC	Mtr.50401.1.S1_at
AC140030_6.1	1.1246	0.046	NAC	Mtr.19019.1.S1_at
CR932967_9.2	2.5307	0.004	PHD	N
AC144541_12.8	2.3144	0.010	PHD	Mtr.18668.1.S1_at
AC135461_11.6	1.2675	0.004	unknown	Mtr.50949.1.S1_at
AC149491_2.7	1.7066	0.038	WD40-like	Mtr.21379.1.S1_at
AC139708_15.2	1.1125	0.045	WD40-like	Mtr.18601.1.S1_at
AC135161_11.7	1.5421	0.040	WRKY	Mtr.52359.1.S1_at
AC135465_24.1	1.4016	0.038	WRKY	N

**Supplemental Table S4.** TFs whose expression levels are regulated by ABA in both wild-type and *latd* mutant roots.

accession no.	TF family	Log <sub>2</sub> FC (WT+ ABA/WT)	Log <sub>2</sub> FC ( <i>latd</i> +ABA/ <i>latd</i> )	Gene Expression Atlas
AC148404_7.7	ABI3-VP1	2.199	1.870	Mtr.49505.1.S1_at
AC146747_7.151	ABI3-VP1	1.280	1.668	Mtr.20853.1.S1_at
AC148404_7.2	ABI3-VP1	9.730	6.511	Mtr.20280.1.S1_at
AC148348_6.1	AP2/EREBP	1.506	2.122	Mtr.20228.1.S1_at
AC146745_18.1	C <sub>2</sub> C <sub>2</sub> (Zn)	2.398	1.354	Mtr.20821.1.S1_at
AC147428_6.2	C <sub>2</sub> C <sub>2</sub> (Zn), GATA	1.245	1.078	N
AC147428_6.6	C <sub>2</sub> C <sub>2</sub> (Zn), GATA	1.832	1.324	Mtr.48513.1.S1_at
AC146329_57.2	C <sub>2</sub> C <sub>2</sub> (Zn), GATA	2.015	2.586	N
AC146720_23.9	GATA	2.278	1.649	Mtr.20716.1.S1_at
AC152184_1.1	CCHC (Zn)	2.921	1.924	N
AC121233_16.1	CCHC (Zn)	12.257	13.084	Mtr.49200.1.S1_at
AC137065_19.2	E2F-DP	1.559	1.298	N
AC126782_22.2	FHA	2.039	1.639	Mtr.15843.1.S1_at
CR538722_9.1	GRAS	-1.428	-2.395	Mtr.44789.1.S1_at
AC152185_1.321	GRAS	-1.248	-1.585	N
AC144724_8.3	HD family	2.064	1.345	Mtr.18980.1.S1_at
AC144759_11.1	JUMONJI	2.125	2.031	Mtr.19107.1.S1_at
AC124959_11.2	KRAB box	1.146	1.880	Mtr.52293.1.S1_at
AC146682_8.1	MYB	1.726	1.427	N
AC146817_10.1	MYB	2.687	1.635	Mtr.21143.1.S1_at
AC149642_2.7	MYB/HD-like	1.647	1.145	Mtr.21051.1.S1_at
AC146747_7.131	NAC	3.901	2.295	Mtr.20852.1.S1_at
AC140544_19.1	NAC	1.562	1.020	Mtr.19055.1.S1_at
AC145753_1.1	NAC	2.695	1.734	Mtr.50038.1.S1_at
AC144541_12.8	PHD	3.649	2.314	Mtr.18668.1.S1_at
AC149491_2.7	WD40-like	3.334	1.707	Mtr.21379.1.S1_at

**Supplemental Table S5.** TFs that are regulated by MtLATD/NIP when ABA is present.

<b>Accession no.</b>	<b>Log2FC (<i>latd</i> ABA/ WT ABA)</b>	<b>p-value</b>	<b>TF family</b>	<b>Gene Expression Atlas</b>
AC148348_6.1	1.6816	0.05	AP2/EREBP	Mtr.20228.1.S1_at
AC137994_19.3	3.8548	0.014	AP2/EREBP	Mtr.46362.1.S1_at
AC149268_1.2	1.3146	0.026	AS2	N
AC148528_15.1	1.8162	0.039	bHLH	N
AC147002_20.2	-1.2410	0.041	BTB/POZ	Mtr.48723.1.S1_at
AC145372_5.1	2.4508	0	bZIP	N
AC135101_39.1	-1.2798	0	C2C2 (Zn)	N
AC135797_13.1	1.1852	0.019	C2H2 (Zn)	N
AC135797_21.1	1.6019	0.033	C2H2 (Zn)	N
AC135797_13.2	1.7573	0.004	C2H2 (Zn)	Mtr.52180.1.S1_at
AC148226_7.1	-2.5199	0.001	CCHC (Zn)	Mtr.49901.1.S1_at
AC123547_15.4	-1.7192	0.022	CCHC (Zn)	N
AC150777_2.1	2.9127	0.015	CCHC (Zn)	N
AC146574_6.1	3.0374	0.013	CCHC (Zn)	Mtr.40781.1.S1_s _at
AC123570_7.1	3.1745	0.008	CCHC (Zn)	N
AC137837_3.3	3.7040	0	CCHC (Zn)	Mtr.42612.1.S1_at
AC147430_9.6	1.9049	0.005	C3H- WRC/GRF	Mtr.21717.1.S1_x _at
AC141113_5.1	2.2034	0.007	C3H- WRC/GRF	Mtr.17466.1.S1_x _at
CR538722_9.1	-2.7376	0.002	GRAS	Mtr.44789.1.S1_at
AC144766_18.5	2.5248	0.003	HD-like	N
AC130809_36.1	1.6438	0.031	JUMONJI	N
AC161107_33.2	2.1673	0.005	MADS	N
AC147712_17.2	-2.8231	0	MYB	N
AC147536_10.1	-2.6666	0.05	MYB	N
AC149493_11.1	1.9936	0.032	MYB	N
AC129092_13.1	-1.2128	0.008	MYB/HD-like	Mtr.16432.1.S1_at
AC135461_11.6	1.0762	0.009	SET	Mtr.50949.1.S1_at
AC137701_25.2	1.5124	0.016	SET	N
CR932967_9.2	1.4613	0.048	PHD	N
AC149801_3.1	-1.5447	0.044	WD-40 like	Mtr.21482.1.S1_at

**Supplemental Table S6.** Primers used in qRT-PCR.

Gene Name	Forward Primer	Reverse Primer	Reference
Mt <i>ENOD11</i>	TAATTGGAGGCTTGT AAGTAG	TAGGGCTTGCTGATA AATCTC	(Zhang, 2013)
Mt <i>RR4</i>	ATGCTTTTGTTCGG GTTA	CTGCACCTTCCTCCA AACAT	
Mt <i>CRE1</i>	CACCACCCTTTGGCT TCTAA	CACTAAGTAGCGGCC TTTCG	
TC165592	ACATGAAGCAGGCTC GTAACA	CCTCCTACGTCTGGC AATGATC	Designed in this study
Mt <i>NCED4</i>	CTTTGCTCCTTGGA GAGTTG	CACCCTCTGGAAAAT CATTCG	
TC177719	GCACGTTTTGTGTTG GTGAC	AGCTTCCTCAAACGC AAGTG	(Zhang, 2013)
Mt <i>IPT3</i>	TGCCTTTGGGTCGAT GTTTC	TCGCAACTCATTAAC CATTCCA	
Mt <i>PREMIR17 1h</i>	CAATTCAGACGAGC CGAAG	GAGCAGAAACAACA CCACTCC	(Ariel et al., 2012)
Mt <i>ERN1</i>	TGTCTCATTGGATTC TCCTCTTGC	TTGGAGCAGAAGCA ACAGCA	(Cerri et al., 2012)
Mt <i>ERN2</i>	CTAGCCATGCTGACA CGACTAAT	ACTGGCTGTGCCAAT ACAGTTAG	
Mt <i>NIN</i>	TGGACAAGGAATAGT AGGAACAG	CTCAATGGAATGGCA ACAGC	(Ding et al., 2008)
Mt <i>RD22</i>	CAAATGTGGGTGTAG GGAAAGGAG	TCAGTGGCAGCATAG TTGTAAGC	
Mt <i>RR1</i>	GCTGAAGTTCACATG CCTGA	CAGCTCCACAGACCA AACAA	(Gonzalez- Rizzo et al., 2006)
Mt <i>NSP2</i>	CTTCTCTGGACACAG CTCCC	AAAGGTGTCCCAGTT GACGA	(Kakar et al., 2008)
Mt <i>UBC9</i>	GGTTGATTGCTCTTC TCTCCCC	AAGTGATTGCTCGTC CAACCC	
Mt <i>PDF2</i>	GTGTTTTGCTTCCGC CGTT	CCAAATCTTGCTCCC TCATCTG	
Mt <i>CLE13</i>	TCAACTTTGCAGGCT CGTAG	TGTAGAAGGCTTCGG CTGTC	(Mortier et al., 2010)

## **CHAPTER FOUR: SUMMARY AND FUTURE DIRECTIONS**

## SUMMARY

### **ABA and Nitrate Transporter MtLATD/NIP Regulate Root Development via Reactive Oxygen Species (ROS)**

Abscissic Acid (ABA) plays an important role in regulating root development, especially in response to environmental stresses. However, how ABA signaling functions in legume root development is largely unknown. ROS are important secondary messengers during ABA-induced stomatal closure in leaves (Wang and Song, 2008), but whether ROS are also downstream of ABA in root development has remained unclear. ABA rescues the root growth defects of *Medicago truncatula latd* mutant that are disrupted in the MtLATD/NIP gene, by increasing its primary root length and restoring its lateral root elongation (Liang et al., 2007). Our study showed that applying ABA decreases the ectopic ROS levels and increases cell elongation in *latd* mutant roots, revealing a mechanism by which ABA restores *latd* mutant root elongation. Pharmacologically decreasing ROS levels can increase cell elongation in *latd* mutant roots, providing evidence that *latd* root elongation defects are at least partially due to the ectopic ROS levels. We found that MtRbohC, a plant NADPH oxidase that contributes to ROS synthesis, has increased expression levels in *latd* roots, and this abnormal expression level can be restored to wild-type levels by exogenous ABA. Silencing MtRbohC expression using RNA interference resulted in increased root elongation and lateral root formation, in both wild-type and *latd* mutants. Together, these results indicate that MtRbohC is an important negative regulator in root development, and is a downstream component of ABA signaling in the control of root elongation and lateral root formation via the nitrate transporter MtLATD/NIP.

## **Transcription Factor (TF) Profiling Reveals New Insights on ABA Signaling Mediated by MtLATD/NIP**

ABA regulation of gene transcription via TFs has been extensively studied in *Arabidopsis*, where a large number of TFs that function downstream of ABA signaling and responses have been identified (Finkelstein et al., 2002; Finkelstein, 2013). No such study has been done in model legume *M. truncatula*, and very few TFs have been shown to be responsive to ABA. In this thesis, we present results of a large-scale TF expression study, using high-throughput qRT-PCR technology, to identify TFs that are transcriptionally regulated by ABA signaling mediated by MtLATD/NIP (Chapter 3). We have identified 20 TFs that exhibit altered expression in *latd* roots, and interestingly, the expression of 60% of these TFs can be restored to wild-type levels by ABA. This has provided novel TF candidates that are likely to be the cause of *latd* root defects, and they could also be the regulators by which ABA rescues *latd* root phenotypes. Therefore, these TF genes are possibly the major regulators in MtLATD/NIP and ABA signaling in root development. We also for the first time identified TFs from various TF families that are regulated by ABA in *M. truncatula* roots, most of which have never been characterized in *M. truncatula*. More importantly, we found that MtLATD/NIP tightly controls the TF set transcriptionally regulated by ABA, demonstrated by the observation that ABA regulates very different TFs when the MtLATD/NIP gene is disrupted. These findings have broadened our understanding of the signaling role of the nitrate transporter MtLATD/NIP, and its pivotal role in mediating ABA signaling. Moreover, TFs regulated by MtLATD/NIP change almost completely when ABA is present, again indicating the close relationships between ABA signaling and nitrate transporter MtLATD/NIP on downstream transcriptional regulation.



## **A Novel Function for MtNSP2 in ABA, MtLATD/NIP and Nitrate Signaling in Root Development**

MtNSP2, *Medicago truncatula* Nodulation Signaling Pathway 2, is a GRAS family TF that plays an essential role in mediating symbiotic nodulation signaling in both bacterial infection in the epidermis and nodule organogenesis in the cortex (Oldroyd and Long, 2003; Kaló et al., 2005; Hirsch et al., 2009). Whether MtNSP2 could have other functions outside of regulating nodulation signaling has been neglected. We were surprised to identify MtNSP2 in our TF profiling as a TF regulated transcriptionally by both ABA and MtLATD/NIP. We found that ABA inhibits the expression of MtNSP2, while MtLATD/NIP stimulates its expression, since the expression level of MtNSP2 is very low in *latd* mutant roots. We also found that nitrate has an inhibitory effect on MtNSP2 expression, likely via a microRNA shown to specifically target the MtNSP2 transcript, miR171h (Lauressergues et al., 2012), which nitrate negatively regulates. We also found that several ABA metabolism-related genes have altered expression in *nsp2-2* mutant roots, indicating MtNSP2 may play a role in regulating ABA homeostasis. Moreover, *nsp2-2* mutants exhibit increased sensitivity to low concentrations of nitrate, displaying altered regulation lateral root formation and elongation, developmental processes also tightly linked with ABA signaling (Signora et al., 2001; Liang and Harris, 2005). In summary, our study has identified a classic symbiotic signaling TF as a novel component of ABA and nitrate signaling, with a key role in regulating ABA-metabolic and responsive gene expression.

### **MtLATD/NIP Functions in Nodulation Signaling by Regulating MtNSP2 Expression**

MtLATD/NIP is required for nodule formation; *latd* mutant nodules are small and white, and lack nitrogen fixation activity (Bright et al., 2005). It has been discussed that MtLATD/NIP is required for the maintenance of nodule meristem function (Bright et al., 2005). Here we describe a signaling role for nitrate transporter MtLATD/NIP much earlier in the nodulation process in the actual initiation of nodulation. More specifically, MtLATD/NIP is required for a very early step in nodulation signaling that is required both for epidermal and for cytokinin-induced cortical signaling via MtNSP2. Without a functional MtLATD/NIP, MtNSP2 expression cannot be induced by cytokinin, and the downstream targets of MtNSP2 also exhibit altered expression patterns. This is also the case during nodulation, where MtNSP2-regulated downstream gene expression remains at a low induction level, or even absent in *latd* mutant roots. Our results have positioned MtLATD/NIP at an early signaling crossroad connecting both bacterial infection in the epidermis and cell division induced by cytokinin in the cortex, upstream of the most important and well-studied TF MtNSP2.

## **FUTURE DIRECTIONS**

### **The Mechanism of ABA and MtLATD/NIP Regulation on Root Elongation**

MtLATD/NIP is a high-affinity nitrate transporter that mediates nitrate uptake in *Xenopus laevis* oocytes, but it also plays important role in root development (Bright et al., 2005; Liang et al., 2007; Bagchi et al., 2012). In *Arabidopsis*, many nitrate transporters have been studied; most them have been shown to not affect root growth except for certain nitrogen conditions. However, *latd* mutant roots exhibit severe cell elongation and meristem defects even under no nitrogen conditions (Bright et al., 2005; Liang et al., 2007; Zhang et al., 2014). Applying nitrate did not rescue the *latd* root phenotype, and the difference in nitrate responses in

*latd* are not significantly from that of wild type (Yendrek et al., 2010). These observations clearly indicate a distinct and crucial role of MtLATD/NIP in root development. In Chapter 2 we have shown that MtLATD/NIP is important for ROS homeostasis in *M. truncatula* roots; many ROS-related genes, especially *MtRboh* genes have altered expression levels when MtLATD/NIP gene is disrupted (Zhang et al., 2014). Decreasing ROS levels by applying ABA or knocking down *MtRbohC* expression can increase cell elongation in the *latd* mutant roots (Zhang et al., 2014). However, some important questions remain: how could altered ROS be linked to cell elongation defects in *latd* roots? What are the other components in ABA and MtLATD/NIP regulation of *MtRboh* and other ROS-related gene expression?

### **Can ROS Lead to Cell Wall Defects in *latd* Mutants?**

ROS are important signaling molecules that can trigger various cellular signaling cascades, but they can also directly modify the cell wall, which leads to changes in growth. Hydrogen peroxide ( $H_2O_2$ ) has been shown to function as an oxidative substrate and promotes crosslinking of the phenolic monomers in the cell wall, such as lignin, which stiffens the cell wall and restricts growth (Schopfer, 1996; Potikha et al., 1999). On the other hand, the highly reactive hydroxyl radicals ( $OH^\cdot$ ), generated from superoxide ( $O_2^{\cdot-}$ ) and  $H_2O_2$  by cell wall peroxidases, can cleave cell wall polysaccharides and result in loosening of the cell wall, allowing growth to occur (Fry, 1998; Schopfer, 2001; Liskay et al., 2004; Francoz et al., 2014). *latd* mutant roots have increased  $O_2^{\cdot-}$  and  $H_2O_2$  (Chapter 2); therefore it is reasonable to speculate that the cell wall in *latd* mutant roots may be affected by elevated ROS. It will be interesting to examine whether lignin deposition could be altered in *latd* roots, especially in the elongation zone, where cells are undergoing elongation. Maybe the elongating *latd* root cells come to an immature stop because

the ectopic ROS accumulation leads to lignin crosslinking in the cell wall. I have begun looking at lignin deposition, and I observed increase lignin deposition in *latd* mutant roots (Appendix II). To further test the correlation between ROS and lignin, it will be important to stain for lignin in *latd* roots with NADPH oxidase inhibitor or transformed with *MtRbohC* RNAi vector. I would also like to check the accumulation or localization of OH<sup>•</sup> in *latd* mutant roots, because the increased expression level of a putative cell wall peroxidase in *latd* roots may contribute to altered OH<sup>•</sup> levels, which could also lead to changes in the cell wall.

### **How Does the Nitrate Transporter MtLATD/NIP Regulate TF Gene Transcription?**

We have shown that 20 TFs exhibit altered expression levels in *latd* mutant roots (Chapter 3). How does the disruption in a gene encoding a nitrate transporter affect gene transcription?

Since MtLATD/NIP transports nitrate (Bagchi et al., 2012), could the altered expression of these TFs be due to disrupted nitrate responses? However, this possibility seems questionable, because plants have multiple nitrate transporters that could maintain or compensate for the loss of MtLATD/NIP. In addition, this could not explain why *latd* mutant roots exhibit altered expression of 20 TFs and have such severe growth phenotypes in the absence of nitrate. Adding nitrate to the growth medium did not rescue *latd* root defects, nor did overexpression of the *Arabidopsis* nitrate transporter AtNRT1.1 (Bagchi et al., 2012), indicating MtLATD/NIP may regulate these TFs and root growth via an unknown pathway.

*latd* mutant roots have increased levels of ROS (Chapter 2). ROS are important signaling molecules that could lead to changes directly in protein kinases and transcription factors (Adler et al., 1999), which can then cause further changes in gene expression. Future study should test if

expression, or activity of these TF genes are also responsive to ROS, by either adding H<sub>2</sub>O<sub>2</sub> or ROS scavenger, or using a chemical inhibitor of ROS synthesis. Another possible way that MtLATD/NIP might modulate TF gene expression is via its role in ABA signaling. ABA can rescue *latd* root growth defects, similar to its effects on ABA-deficient mutants (Cheng et al., 2002; Bright et al., 2005). If *latd* roots were experiencing lower levels of ABA, this could lead to disruption of ABA signaling and downstream gene transcription changes. To test this, we could either use an ABA biosynthesis inhibitor to decrease endogenous ABA levels and check the expression of these TFs, or we could transform the *Arabidopsis* *abi1-1* construct into *M. truncatula* roots to block ABA signaling (Ding et al., 2008), then ask whether these TFs exhibit same changes as in *latd* mutant roots.

### **What are the Transcription Factors Downstream of ABA, MtLATD/NIP and ROS in Root Development?**

We have identified TFs transcriptionally regulated by ABA and MtLATD/NIP in Chapter 3. Both applying ABA and disruption of the *MtLATD/NIP* gene lead to altered *MtRboh* gene expression. To identify downstream TFs that may function in ABA and MtLATD/NIP-regulated *MtRboh* gene expression, it will be useful to perform a second-round screen on ABA and MtLATD/NIP-regulated TFs and check if any of them are also ROS-responsive. Since *latd* root growth defects are partially due to the ectopic ROS levels, the TFs that are regulated by ABA, MtLATD, and ROS might be important regulators in this signaling pathway. Sixty percent of the TFs with altered expression levels in *latd* roots can be restored to wild-type levels by applying 10  $\mu$ M ABA (Chapter 3). ABA also rescues the ectopic ROS accumulation and *MtRbohC* expression in *latd* roots (Chapter 2), thus it would be a good first step to screen through these ABA-rescued

TFs for ROS-regulation. If there are TFs that meet this criterion, it would be interesting to test if they could possibly bind to the promoter of *MtRboh* genes and regulate their expression.

### **Can the Nitrate Transporter MtLATD/NIP Transport Other Molecules?**

MtLATD/NIP belongs to the large NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER (NRT1/PTR) family, which has been recently renamed the NPF family, for **NRT1/PTR FAMILY** (Léran et al., 2014). Members of the NPF family have been shown to transport a wide range of molecules, such as nitrate, glucosinolates and dipeptides, as well as the plant hormones auxin and ABA (Krouk et al., 2010; Kanno et al., 2012; Léran et al., 2014). The *Arabidopsis* dual-affinity nitrate transporter AtNRT1.1/NPF6.3 also transports auxin in a nitrate-dependent manner: AtNRT1.1/NPF6.3 facilitates auxin shootward transport in absence of nitrate, in order to limit auxin accumulation at the LR tip, which slows down LR elongation (Krouk et al., 2010). When nitrate is present, transport of auxin by AtNRT1.1/NPF6.3 is blocked, resulting in auxin accumulation in the LR tip, stimulating the LR to grow (Krouk et al., 2010). The low affinity nitrate transporter AtNRT1.2/AT1/NPF4.6 can transport both nitrate and ABA, but nitrate does not seem to compete with ABA as a substrate (Huang et al., 1999; Kanno et al., 2012; Kanno et al., 2013). Since *latd* mutants exhibit ABA-insensitive phenotypes in ABA-induced stomatal closure and ABA-inhibited seed germination, and can be rescued by application of ABA to the root, it is possible that this nitrate transporter can also transport ABA, and that some of the defects in the *latd* mutant may be due to an inability to transport ABA. To test this possibility, a functional characterization of ABA transport activity needs to be done, and a modified yeast two-hybrid assay could also be useful: GAL4 Activation domain (AD) fused with ABI1, DNA Binding domain (BD) fused with ABA receptor PYR1, with LacZ as the reporter of the

interaction between PP2C ABI1 and ABA receptor PYR1 (Kanno et al., 2012). If cells expressing MtLATD/NIP growing in medium with ABA have higher  $\beta$ -Gal activity, this indicates MtLATD/NIP have the ABA uptake activity. On the other hand, using radioactive  $^3\text{H}$ -ABA can also test the ability of MtLATD/NIP to transport ABA. Such experiments can also be done with nitrate supplementation, which will help to answer the question of whether nitrate affects ABA transport by MtLATD/NIP.

### **Lessons from Arabidopsis Nitrate Transporters?**

Many nitrate transporters in *Arabidopsis* have been well characterized: AtNRT1.1 functions as a nitrate sensor via phosphorylation by two Calcium-Dependent Protein Kinases (CDPKs) (Ho et al., 2009). It has recently been shown that phosphorylation can switch AtNRT1.1 from homodimers to monomers; in this way AtNRT1.1 changes from a low-affinity to a high-affinity nitrate transporter (Parker and Newstead, 2014; Sun et al., 2014). Although MtLATD/NIP does not exhibit dual affinity in nitrate transport (Bagchi et al., 2012), it will still be interesting to see if such post-translational regulation exists for MtLATD/NIP. Since CDPKs have been shown to be involved in ABA signaling (Zhu et al., 2007; Asano et al., 2012), it is possible that CDPK-triggered MtLATD/NIP phosphorylation may be one way to transduce the ABA signal and modulate downstream gene expression and root growth. Moreover, transcription factors have been shown to be the transcriptional regulators of some nitrate transporters. The bZIP TF TGACG SEQUENCE-SPECIFIC BINDING PROTEIN 1 and 2 (TGA1 and TGA2) bind to the promoter of AtNRT2.1 and AtNRT2.2, two genes encoding high-affinity nitrate transporters, in a nitrate-dependent manner (Alvarez et al., 2014). TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1-20 (TCP20), can bind to

the promoter of AtNRT1.1 and AtNRT2.1 and regulate their expression, and further functions systemically in promoting LR development in nitrate rich areas (Guan et al., 2014). To further understand the function of MtLATD/NIP, it is important to search for proteins that could either interact with the MtLATD/NIP protein itself, or bind to the MtLATD/NIP promoter and regulate its expression. Thus, I would propose a further experiment aiming at looking for MtLATD/NIP interacting proteins. Using yeast two-hybrid assay may be problematic because MtLATD/NIP is a membrane protein; an alternative method, the yeast split ubiquitin system (SUS) is more suitable for studying membrane interacting proteins (Stagljar et al., 1998; Obrdlik et al., 2004; Orsel et al., 2006). It would also be interesting to search for potential TFs that could bind to the promoter of MtLATD/NIP, using the yeast one-hybrid assay (Ouwerkerk and Meijer, 2001). In this way, MtLATD/NIP interacting or regulatory proteins that may be involved in ABA or nitrate signaling can be identified, which could deepen our understanding of the mechanism of how MtLATD/NIP regulates gene expression.

Together, future studies on the mechanism by which MtLATD/NIP mediates ABA signaling in *M. truncatula* could focus on three major approaches: a) closely examine cell wall composition in different developmental stages of *latd* mutant roots and examine how ABA and ROS affect the cell wall, b) characterize the TFs that regulated by ABA, MtLATD/NIP, nitrate and ROS, and c) explore the possibility of MtLATD/NIP in transporting ABA biochemically, as well as whether nitrate plays any role in the regulation of MtLATD/NIP ABA transport.

### **A Signaling Role for MtLATD/NIP in Nodulation**



Disruption of the *MtLATD/NIP* gene leads to defects in the formation of functional symbiotic nodules (Veereshlingam et al., 2004; Bright et al., 2005). Bacterial infection of *latd* mutant roots is incomplete; ITs can be formed but don't progress far into the root or nodule tissue. Similarly, cell division occurs in the cortex to form a nodule primordium, but nodules on *latd* roots are small and unable to fix nitrogen (Bright et al., 2005). My results showed that *MtLATD/NIP* is not only required for the later steps of nodule morphogenesis and nitrogen fixation, but is also required for early nodulation signaling in both the epidermis and the cortex, mediated by the *MtNSP2* TF and its downstream targets (Chapter 3). *MtNSP2* expression levels remain very low in *latd* roots, and its downstream targets *MtERN1*, *MtNIN* and *MtENOD11*, which are involved in rhizobial infection in the epidermis, all exhibit decreased induction or altered temporal pattern in response to *S. meliloti* inoculation (Chapter 3). Cytokinin, which is a major hormone that regulates cell division in the cortex and triggers the cortical nodulation pathway via *MtNSP2*, cannot induce *MtNSP2* expression in *latd* mutant roots, and thus the cortical nodulation pathway is not activated (Chapter 3). These observations indicate that *MtLATD/NIP* acts upstream of *MtNSP2* during early nodulation signaling, and is required for crosstalk between cytokinin and nodulation signaling via *MtNSP2*. But how does *MtLATD/NIP* play such an important role in nodulation signaling?

### **How does *MtLATD/NIP* Positively Regulate Nodulation?**

I have shown that *MtLATD/NIP* is required for induction of genes involved in both rhizobial infection in the epidermis and nodule organogenesis in the cortex, suggesting that *MtLATD/NIP* plays a positive role in both epidermal and cortical signaling pathways of nodulation. This seems to contradict the fact that *MtLATD/NIP* is a nitrate transporter, whose

substrate is an inhibitor of nodulation (Stephens and Neyra, 1983; Streeter, 1985; Malik et al., 1987). However, as discussed above, it is possible that MtLATD/NIP functions as a nitrate sensor that is sensitive to low nitrate concentrations, which could induce nodulation.

It has been shown that the expression of MtLATD/NIP can be up-regulated by exogenous cytokinin (Yendrek et al., 2010). I found that *latd* mutant roots are defective in cytokinin induction of nodulation genes, but not in the cytokinin-induced expression of cytokinin receptor CRE1, Type-B Response Regulator RR1, and Type-A Response Regulator RR4 (Chapter 3). Thus, I expect MtLATD/NIP to function downstream of CRE1 and RR1, but in a separate pathway from RR1 inducing RR4. Since MtNSP2 is induced by cytokinin via RR1 binding to its promoter, it will be interesting to check if MtLATD/NIP is required for such interaction. Also, could RR1 bind to the MtLATD/NIP promoter and induce its expression? These possible future studies could help us determine how MtLATD/NIP is involved in cytokinin signaling.

The disruption in cytokinin signaling in the cortex of *latd* mutant roots does not fully explain why the epidermal signaling pathway is also defective. Infection thread (IT) progression is defective and the IT seems to be abnormally thick with a wide or irregular shape in *latd* or *nip-1* mutant roots (Veereshlingam et al., 2004; Bright et al., 2005). Since *latd* roots have ectopic ROS levels and increased expression of several MtRboh genes (Chapter 2), it is also a possibility that the increased ROS in *latd* roots may affect nodulation. Studies have shown that ROS are also signaling molecules in nodulation processes; the roles of ROS seem to be complicated at different stages during nodulation (Lee et al., 2005; Pauly et al., 2006; Cardenas et al., 2008; Marino et al., 2011). Thus, decreasing ROS chemically in *latd* roots may not be ideal, but it will be interesting to knock down the expression of the MtRboh genes whose expression levels are increased in *latd* roots and check whether there would be an effect on the shape or progression of IT in *latd* roots.

## **Spatiotemporal Pattern of MtLATD/NIP mRNA and Protein During Nodulation**

To date, the expression pattern of MtLATD/NIP has only been shown to be high in root tips and nodule meristem (Yendrek et al., 2010). However, to better understand how MtLATD/NIP functions in nodulation, it is important to examine the following questions: where in the *M. truncatula* root is MtLATD/NIP expressed during nodulation? Which cell types express MtLATD/NIP? Where is the MtLATD/NIP protein localized in the roots? Does the localization of transcripts or proteins alter in response to *S. meliloti* inoculation? Using the MtLATD/NIP promoter:GFP/GUS fusion, and MtLATD/NIP promoter:MtLATD/NIP-HA vector, or developing an antibody against MtLATD/NIP will help answer those questions.

## **Could MtNSP2 Have a Novel Function in Coordinating Root Development with Nodulation?**

MtNSP2, a GRAS family TF, is the most important TF that is required not only in Nod factor signaling, but also in Myc factor signaling in mycorrhization (Kaló et al., 2005; Liu et al., 2011; Maillet et al., 2011). It is the last TF in the common symbiotic pathway (Oldroyd, 2013). MtNSP2 can form different heterocomplexes with different GRAS family TFs: it interacts with MtNSP1 to induce nodulation gene expression, while it associates with REQUIRED FOR ARBUSCULAR MYCORRHIZATION 1 (MtRAM1) in mycorrhizal signaling (Hirsch et al., 2009; Gobbato et al., 2012). Interestingly, both Nod factor and Myc factor can induce the formation of lateral roots, as well as lateral root elongation, via NSP2/NSP1 and NSP2/RAM1 respectively (Oláh et al., 2005; Maillet et al., 2011; Gobbato et al., 2012). These results indicate the role of MtNSP2 in regulating lateral root development during nodulation or mycorrhization,

but the mechanism of how MtNSP2 stimulates lateral root formation under these symbiotic conditions is not clear. Moreover, MtNSP2 is a direct target downstream of cytokinin signaling (Ariel et al., 2012), and is required for strigolactone (SL) biosynthesis by regulating SL biosynthetic gene expression in both *M. truncatula* and *Oryza sativa* (rice) (Liu et al., 2011), suggesting MtNSP2 plays multiple roles that intersect with different hormone signaling, and the function of MtNSP2 is not limited to legume species. Interestingly, using NSP2 homolog in rice has successfully rescued nodulation phenotype in another model legume species, *Lotus japonicas* (Yokota et al., 2010). This cross-species complementation indicates that the function of NSP2 is conserved in non-legume species; however, the exact role of NSP2 in non-symbiotic roots in legumes, or that in non-legume species such as *Arabidopsis* and rice, has not been identified.

### **Is MtNSP2 Involved in ABA and Nitrate Signaling Crosstalk?**

I have shown in Chapter 3 that MtNSP2 is under the regulation of ABA, nitrate, and MtLATD/NIP in non-symbiotic legume roots. Interestingly, *nsp2-2* mutant roots appear to have altered nitrate responses in LR elongation (Chapter 3). I have observed that under low nitrate concentrations (250  $\mu$ M), *nsp2-2* mutants have increased LR density and total LR length, while wild-type plants do not respond to nitrate at this concentration (Chapter 3). It seems that *nsp2-2* mutants have increased sensitivity only to low concentrations of nitrate, indicating that the wild-type function of MtNSP2 must be to negatively regulate low nitrate stimulation of LR development. To further investigate the role of MtNSP2 in nitrate-regulated LR development, I would like to test the LR responses of *nsp2-2* mutants to locally applied nitrate from low to high concentrations by a nitrate patch experiment, since LR formation responds differently to a local patch of nitrate than to global nitrate (Zhang and Forde, 2000). It will also be interesting to

overexpress MtNSP2 in transgenic roots and then check the LR responses to nitrate, as a comparison to the *nsp2-2* loss-of-function mutants. However, since MtNSP2 transcript is a target of a microRNA, miR171h (Lauressergues et al., 2012), it may be important to overexpress a miR171-resistant version of MtNSP2.

### **What is the Function of NSP2 in Non-Legume Species?**

Another potentially interesting direction for exploring the function of NSP2 in non-symbiotic roots would be to use non-legumes species, such as *Arabidopsis*, which has a homolog of MtNSP2, AtSCL26 (At4g08250) (Lee et al., 2008; Delaux et al., 2013). I have begun to look for T-DNA insertion mutant lines of the *nsp2* mutants in *Arabidopsis*. If a mutant could be successfully isolated, further experiments would be to check root growth phenotypes in ABA and nitrate. At the same time, since MtNSP2 and OsNSP2 both regulate the biosynthesis of strigolactone (Liu et al., 2011), which is shown to inhibit LR development and increase root hair elongation in *Arabidopsis* (Kapulnik et al., 2011), it will be exciting to see whether ABA and SL signaling crosstalk exists and whether NSP2 is involved in such crosstalk.

### **Could MtNSP2 Be the Decision Maker of Initiating a LR or a Nodule in *M. truncatula*?**

*M. truncatula* roots develop two types of lateral root organs, LRs and nodules, which were thought to originate from different founder cells (Ding and Oldroyd, 2009), but also exhibit many similarities in the developmental processes. Recently, a precise nodule fate map for *M. truncatula* has been published, demonstrating that the mitotic activation of root cells by rhizobium starts in the pericycle and extends to the cortical cell layers, with the third cortical cell

layer becoming the nodule meristem (Xiao et al., 2014). LR formation starts from cell divisions in the pericycle, then extends to the endodermis, with inner cortical cells dividing only at later stages, forming the external part of the LR primordia (Herrbach et al., 2014). The number of LRs and nodules needs to be balanced; the initiation of nodule primordia leads to a suppression of LR emergence (Nutman, 1948; Ding and Oldroyd, 2009). The balance of LRs and nodules is maintained by plant hormones: studies examining cytokinin signaling and response found that cytokinin promotes nodule organogenesis while it inhibits LR development (Lohar et al., 2004). The cytokinin response is specifically shut off in the pericycle cells undergoing division during forming of the LR primordium, but is activated in dividing cortical cells during nodule primordium formation (Lohar et al., 2004). Knocking down cytokinin receptor *MtCRE1* expression resulted in a decreased number of nodules but increased number of LRs (Gonzalez-Rizzo et al., 2006). ABA, as an antagonistic partner to cytokinin, inhibits nodulation and stimulates LR formation (Liang et al., 2007; Ding et al., 2008). Blocking ABA responses in *M. truncatula* roots using the dominant *Arabidopsis abi1-1* mutation, leads to increased nodulation and higher induction by cytokinin of the expression of *EARLY NODULIN 40*, which is stimulated in the dividing cells during nodule primordium formation (Ding et al., 2008). In response to inoculation, the ABA/cytokinin ratio in a soybean supernodulating mutant is less than that of wild-type (Caba et al., 2000; Ding and Oldroyd, 2009), suggesting that reaching a specific ratio of ABA to cytokinin is crucial for the initiation of LR or nodules (Caba et al., 2000; Ding and Oldroyd, 2009).

Cytokinin induces the expression of *MtNSP2* (Ariel et al., 2012), and I have shown that ABA represses expression of *MtNSP2* (Chapter 3), which is consistent with the observation that ABA and cytokinin act antagonistically in nodulation. ABA and cytokinin also have opposite effects on LR initiation; however, whether *MtNSP2* is involved in ABA and cytokinin crosstalk in LR development is not clear. *nsp2-2* mutants grown on standard medium without nitrogen do

not have obvious difference in LR formation, compared to wild type, suggesting that the role of MtNSP2 in LR development may be only under specific conditions. I have shown that *nsp2-2* mutants have increased sensitivity to low nitrate concentrations in stimulating LR formation (Chapter 3). Is it possible that under low nitrate conditions, MtNSP2 functions to mediate cytokinin and ABA signaling crosstalk to help make the decision of whether to form a nodule instead of a LR? Could MtNSP2 regulate the balance between cytokinin and ABA for legumes to decide which organ to initiate?

Thus, I propose a future plan to investigate the possibility of MtNSP2 regulating cytokinin and ABA ratio in *M. truncatula* roots, under no nitrate and low nitrate, as well as under high concentrations of nitrate. A previous study looking at the global gene expression profile of uninoculated *nsp2-2* roots revealed that many ABA metabolism-related and nitrate signaling genes have altered expression levels in the Nod factor susceptible zone (growing root hair zone) of *nsp2-2* mutant roots (Liu et al., 2011), leading to the possibility that ABA levels in *nsp2-2* mutant roots could be altered. I have also verified that ABA-metabolism-related gene expression is down-regulated in *nsp2-2* whole root tissue, and that the expression of these ABA-metabolism genes in *nsp2-2* roots is not responsive to ABA, while in wild-type roots, ABA can strongly inhibit their expression, suggesting the regulation of ABA metabolism may be disrupted in the *nsp2-2* mutants (Chapter 3). It will be necessary to accurately measure how much ABA and cytokinin are in *nsp2-2* mutants, and whether inoculation with *S. meliloti* changes the levels of these hormones. It will also be interesting to explore whether different nitrate conditions have any effect on ABA and cytokinin levels, or the ratio of ABA/cytokinin in *nsp2-2* roots compared to wild type.

Moreover, it is worth looking more deeply into the role of MtNSP2 in ABA signaling. Since the inhibitory effect of ABA on MtNSP2 expression I observed is due to exogenous ABA application, it will also be informative if we can block ABA synthesis or signaling, then check the

expression of *MtNSP2*. In this way, the effect of physiological level of ABA on *MtNSP2* expression can be determined. Fluridone has been used as an ABA biosynthesis inhibitor; however, it is an inhibitor of carotenoid biosynthesis, and thus has detrimental effects on plant growth (Gamble and Mullet, 1986). Abamine (stands for ABA biosynthesis inhibitor with an amine moiety) on the other hand, may be a much better ABA inhibitor, because it is designed as specific to 9-cis-epoxycarotenoid dioxygenase (NCED), the first rate-limiting enzyme in ABA biosynthetic pathway (Qin and Zeevaart, 1999; Han et al., 2004). Thus, by specifically blocking ABA biosynthesis chemically then checking the expression of *MtNSP2*, we can answer the question of how reducing ABA levels affects *MtNSP2* expression. Another approach would be using an ABA biosynthesis or signaling mutants and check whether they have altered *MtNSP2* expression. Such mutants can be isolated from the TILLING population or the *Tnt1* mutant population, but their role in ABA synthesis would first need to be demonstrated (Tadege et al., 2008; Carelli et al., 2013).

Together, the experiments proposed above will help us to understand how *MtNSP2* is regulated by endogenous ABA, whether *MtNSP2* plays a role in ABA metabolism, and whether *MtNSP2* is involved in reading the ratio of ABA to cytokinin, which could be the decision point for initiating a LR or a nodule in *M. truncatula*.



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## **APPENDICIES**

**I. MtlATD/NIP AND ABA REGULATE ROOT MERISTEM-SPECIFIC GENE EXPRESSION**

**II. CELL WALL-RELATED GENE EXPRESSION AND LIGNIN DEPOSITION ARE REGULATED BY MTLATD/NIP**

**III. FUNCTIONAL CHARACTERIZATION OF TRANSCRIPTION FACTORS DOWNSTREAM OF ABA AND MTLATD/NIP SIGNALING PATHWAY**

**IV. CHARACTERIZATION OF A ROOT HAIR MUTANT OF *MEDICAGO TRUNCATULA***

## **APPENDIX I. MtLATD/NIP AND ABA REGULATE ROOT MERISTEM-SPECIFIC GENE EXPRESSION**

### **INTRODUCTION**

Root development is a highly programmed process, beginning with embryogenesis and continuing throughout the life of adult plants. Plant root growth is the result of cell division and cell elongation, which occur in the meristem region at the tip of the roots. The root apical meristem (RAM) contains undifferentiated stem cells, which give rise to different cell types that have distinct functions in the root (Petricka et al., 2012). The specification of a root is established during embryogenesis, and this process is regulated by various hormones, with auxin being the major regulator (Lau et al., 2012). The RAM established during embryogenesis contains a set of stem cells, or initial cells, surrounding the quiescent center (QC), which is referred to as the stem cell niche (Heidstra and Sabatini, 2014). The initial cells can divide and give rise to new cells, while the QC is a set of less mitotically active cells that is essential for specification of the stem cell niche and the maintenance of the stem cells being undifferentiated (Petricka et al., 2012). The initial cells in the RAM produce new cells, which will then undergo three distinct developmental phases to reach their maturity: proliferation in the meristematic zone (MZ), subsequent elongation in the elongation zone (EZ), and finally, in the differentiation zone, they will acquire their specialized characteristics and function (Petricka et al., 2012).

Plant hormones play critical roles in maintaining RAM function and regulating root growth. Auxin is one of the best-studied plant hormones to function in the RAM. A current model is that the shoot-derived auxin moves rootward through the vascular system, and is laterally redistributed once it reaches the root cap, to the external cell types, and then is transported back

into the shoot via the PIN proteins (Petricka et al., 2012). The auxin maximum in the root is localized in the stem cell niche, and the auxin gradient leads to root responses mediated by various transcription factors (Petricka et al., 2012).

ABA also positively regulates RAM function by promoting the quiescent signature of the QC cells and inhibiting stem cell differentiation in the RAM (Zhang et al., 2010). However, the mechanism of how ABA regulates RAM function is not well studied. ABA has been long recognized as a growth inhibitor, but it clearly also plays a positive role in root growth, because some ABA-deficient mutants have root growth defects that can be restored by adding ABA (Cheng et al., 2002). In *Medicago truncatula*, ABA can rescue the root growth defects in the *latd* mutants promoting root elongation (Liang et al., 2007). MtLATD/NIP has been shown to transport nitrate in *Xenopus* oocytes (Bagchi et al., 2012), and the MtLATD/NIP gene is preferentially expressed in the root and nodule meristem (Yendrek et al., 2010). The *latd* mutant has severe defects in the root meristem organization and root length in both primary and lateral roots, which can be rescued by exogenous ABA application (Bright et al., 2005; Liang et al., 2007). Root growth is the result of two events that take place in two adjacent developmental zones in the root apical meristem: cell division in the MZ, and cell elongation in the EZ. We have shown in Chapter Two that *latd* mutant roots have defects in cell elongation (Zhang et al., 2014). Since *latd* mutant roots also exhibit severe defects in RAM organization, we wondered whether the meristem-specific gene expression is also affected in *latd* mutant roots, and how ABA plays a role in regulating the expression of these genes. Here I show that many genes involved in PLT and SHR/SCR pathways functioning in RAM patterning and function do not exhibit any difference in their expression levels in *latd* mutants, indicating MtLATD/NIP may act in a separate pathway in regulating RAM function. Also, ABA appears to only affect the expression

of Mt*PLT1* and Mt*PLT2*, but not Mt*SHR* or Mt*SCR*, which suggests that ABA may only regulate the PLT signaling pathway in the RAM of *M. truncatula*.

## RESULTS AND DISCUSSION

### RAM-Specific Gene Expression is Not Altered in *latd* Mutant Root Tips

The Arabidopsis stem cell niche is maintained by the QC via a set of specifically expressed genes, regulated by two separate signaling pathways functioning in maintaining the QC identity and the stem cell niche: the PLETHORA (PLT) pathway and the SHORTROOT/SCARECROW (SHR/SCR) pathway (Petricka et al., 2012). *PLT1* and *PLT2* encode AP2/EREBP-like transcription factors, and they are first expressed in the basal region in the embryo, then in the embryonic root primordium, and later in the RAM stem cell niche, including the QC and its surrounding cells, as well as the columella root cap cells (Aida et al., 2004). PLT expression is regulated by auxin; disruption in *PLT1* and *PLT2* genes lead to cell proliferation defects, but has no effect on cell elongation, indicating the short root phenotype in *plt1* and *plt2* mutants is due to reduced cell division. We wondered whether the RAM defects in the *latd* mutant might be due to altered expression of the PLTs. To test this, I checked the gene expression of Mt*PLT1* (GenBank accession number AY506549) and Mt*PLT2* (GenBank accession number AY506550) (Imin et al., 2007) in 7-day old *latd* mutant root tips. I did not observe any significant changes in either *PLT1* or *PLT2* gene expression (Fig. 1A), indicating that at this developmental stage the RAM in *latd* mutants is not defective in *PLT1* and *PLT2* expression, even though at this stage the *latd* root growth rate is already slower than that of wild type (Bright et al., 2005).

The other pathway controlling RAM patterning and function in Arabidopsis, is the SHR/SCR pathway. SHR and SCR are GRAS family transcription factors, and the stele-expressed SHR moves into the endodermis to turn on SCR, which is required for stem cell specification and RAM organization (Di Laurenzio et al., 1996; Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003). Mutants of both *SHR* and *SCR* genes exhibit a short root phenotype, although this phenotype is much more extreme in *shr* mutants (Di Laurenzio et al., 1996; Helariutta et al., 2000). We wondered whether the expression of MtSHR and MtSCR in *latd* RAM might be correlated with the *latd* short root phenotype. *SHR* and *SCR* expression did not show any significant difference between 7-day *latd* and wild-type root tips (Fig. 1B). *WUSCHEL-RELATED HOMEODOMAIN 5* (*WOX5*) acts downstream of the SHR/SCR pathway, but not the PLT pathway, in maintaining stem cell specificity (Sarkar et al., 2007; Petricka et al., 2012). *WOX5* is specifically expressed in the QC; it functions in maintaining stem cells in the RAM and prevents them from differentiating (Sarkar et al., 2007). Mutants lacking a functional *WOX5* in Arabidopsis have a disorganized RAM (Sarkar et al., 2007). Since the RAM in *latd* mutant roots becomes disorganized as development progresses, we wondered if this phenotype could be due to *WOX5*. Again, I did not observe any difference in MtWOX5 expression in young *latd* mutant root tips (Fig. 2A). Since the PLTs and SHR/SCR control root patterning, *latd* roots were cross-sectioned and they appear to have normal tissue patterning (Fig. 1C). The MtSHOOT MERISTEMLESS (MtSTM) and MtBABYBOOM (MtBBM), which are also expressed in the root meristem (Imin et al., 2007), have normal expression levels in *latd* root tips (Fig. 2B and C). These results suggest that at 7 days, *latd* root tips have normal expression of RAM genes that play major role in the PLT and SHR/SCR signaling pathway, despite of the fact that *latd* roots are already shorter than wild-type roots.



The *latd* root growth rate decreases as the plants get older, and gradually arrests at approximately 15 days after germination (Bright et al., 2005). Root tip sections have revealed that the 24-day old *latd* root meristem is disorganized, with the stem cell niche very hard to identify (Liang et al., 2007). The RAM in *latd* mutants goes through meristem exhaustion as the development proceeds. Young *latd* roots at 7 days are still growing, although at a slightly decreased growth rate relative to wild-type roots (Bright et al., 2005), with a normal patterned RAM. However, the root length of *latd* mutants is already shorter than that of wild type at the same age. Here we show that at 7-days post germination, *latd* root tips have normal expression levels of MtPLT1, MtPLT2 and MtSCR, MtWOX5. Combining gene expression data with the fact that at this stage the *latd* RAM appears to be normal, suggests that the meristem arrest is a much later event and may not be the direct cause that leads to primary root elongation defects at early age. However, it is still possible that the spatial expression pattern of these RAM-specific genes is altered in *latd*, which would not be shown from qRT-PCR approach. It will be interesting to use promoter: GUS fusions of various meristem-expressed genes, or *in situ* hybridization to determine the exact localization of meristem gene expression in the *M. truncatula* root tip. Also, it will be interesting to investigate whether expression levels of RAM genes alter at later developmental stages than the ones examined here.

Cells actively proliferate in the MZ of the RAM. Since *latd* roots do not exhibit any significant defects in either RAM organization or gene expression levels, we wondered whether cell proliferation in the *latd* root meristem is altered. MtCYCLIN B-1,2 (AJ621872) has been used as a cell cycle marker, expressed only in the G2 phase and early M phase (Gimeno-Gilles et al., 2009). In 7-day old *latd* root tips, the expression of MtCYCLIN B-1,2 did not show any difference from wild-type (Fig. 2D). It is possible that in young *latd* mutant roots, cell division can occur normally. We have shown that *latd* mutant roots have increased levels of reactive oxygen species

(ROS) that is correlated with the root elongation defects in Chapter 2 (Zhang et al., 2014). ROS homeostasis plays important role in regulating cell proliferation and cell elongation. In *Arabidopsis*, superoxide ( $O_2^{\cdot-}$ ) is mainly present in the MZ and hydrogen peroxide ( $H_2O_2$ ) is in the EZ; the balance of  $O_2^{\cdot-}$  and  $H_2O_2$  in the transition zone (TZ) is critical for the shifting from cell division to elongation; this transition is under the regulation of a bHLH transcription factor, UPBEAT 1 (Tsukagoshi et al., 2010). *latd* mutant roots have increased levels of both types of ROS molecules, and whether this also contributes to the meristem arrest is unknown. ROS molecules are toxic and ROS burst has been shown to cause cell death (Swanson and Gilroy, 2010). Whether the cells in the *latd* RAM are experiencing premature cell death would also be an interesting question to look into.

### **ABA Regulates Expression of A Subset of RAM Genes**

ABA can rescue the *latd* root meristem defects in two different ways: ABA not only rescues the organization and patterning of *latd* RAM, but also maintains the function of the *latd* RAM as a center of cell proliferation and cell elongation. ABA plays a positive role in RAM function by promoting the QC quiescence as well as repressing stem cell differentiation (Zhang et al., 2010). Genetic evidence has revealed that in *Arabidopsis*, ABA inhibits QC division through the Protein Phosphatase 2C ABA INSENSITIVE 1 and 2 (ABI1 and ABI2), and transcription factors ABA INSENSITIVE 3 (ABI3) and ABA INSENSITIVE 5 (ABI5), and it represses stem cell differentiation by inducing the expression of *WOX5* and *MONOPTEROS* (Zhang et al., 2010). We wondered whether ABA also regulates RAM gene expression in *M. truncatula* root tips and whether ABA rescues *latd* RAM defects via regulating important RAM genes. We found that in wild-type root tips, ABA inhibits the expression of MtPLT1 and MtPLT2 (Fig. 1A), but has no effect on MtWOX5, MtSHR and MtSCR (Fig. 1B and 2A). ABA has no statistically

significant effect on either *MtBBM* or *MtSTM* (Fig. 2B and 2C). In *latd* root tips, ABA only significantly down-regulates the expression of *MtPLT2* (Fig. 1A). Also, ABA down-regulates the expression of *CYCLIN B-1,2* in the Arabidopsis RAM, indicating its negative role in controlling cell division in the MZ (Zhang et al., 2010). I did not observe similar effect of ABA in *M. truncatula* root tips, where ABA did not exhibit any significant effect on *MtCYCLIN B-1,2* expression (Fig. 2D). Clearly the expression pattern of RAM genes regulated by ABA in *M. truncatula* is different from that in Arabidopsis, and this could be due to the different duration of ABA treatments and different ABA concentrations used: in our experiments, we used 7-day continuous 10  $\mu$ M ABA treatment, while in Zhang et al., tissue was harvested after 4, 8 and 24 hour 1  $\mu$ M ABA treatments (Zhang et al., 2010). It is also possible that ABA regulation in *M. truncatula* RAM is different from that in Arabidopsis. ABA stimulates the formation of lateral roots in *M. truncatula*, while inhibits that in Arabidopsis, and this phenomenon appears to have been acquired near the base of the Leguminosae (Liang and Harris, 2005). Since *M. truncatula* makes two types of meristems in roots, the root meristem and the nodule meristem, it will be interesting to examine the genes that are involved in the formation and function of these two meristems. To date, no study has looked at RAM patterning genes in the PLTs and SHR/SCR signaling pathways in the patterning of nodule meristem. Are the mRNAs of these genes even present in nodule meristem? Studies have revealed that cytokinin and auxin play major roles in nodule organogenesis, and the expression of PLT genes as well as SHR and SCR is auxin-responsive, and highly connected with auxin signaling in Arabidopsis. Whether these signaling pathways are present in *M. truncatula* RAM and nodule meristems will be very interesting to ask. Moreover, the function of ABA in RAM of *M. truncatula* is not clear, and its role in nodule meristem is completely unknown. It is worthwhile to explore the expression pattern of the RAM patterning genes in nodule meristem, as well as the effect of ABA on their expression. Such

experiments will provide insight into the regulation of ABA on different types of RAM between legume and non-legume species.

## APPENDIX II. CELL WALL-RELATED GENE EXPRESSION AND LIGNIN DEPOSITION ARE REGULATED BY MTLATD/NIP

### INTRODUCTION

*Medicago truncatula latd* mutants have defects in root growth and development, which can be observed starting at a very early stage, within a few days after germination (Bright et al., 2005). In Chapter 2 we have shown that epidermal cells in young *latd* root are significantly shorter, and later on, epidermal cells in *latd* roots also exhibit cell shape change (Zhang et al., 2014). We have also found that Reactive Oxygen Species (ROS) levels are increased in *latd* roots, and ectopic superoxide ( $O_2^{\cdot-}$ ) accumulation is found at the cortical cell junctions, in the direction of cell elongation (Zhang et al., 2014). The Arabidopsis mutant *rhd2*, which is defective in the major ROS-generating enzyme NADPH oxidase, RESPIRATORY BURST OXIDASE HOMOLOG C (AtRbohC), has normal root hair initiation, but these root hairs cannot elongate (Foreman et al., 2003). In wild-type plants, ROS accumulates at the growing root hair tip, but this pattern is absent in *rhd2* mutant root hairs, suggesting  $O_2^{\cdot-}$  accumulation at the growing root hair tip is required for normal cell elongation (Foreman et al., 2003). *rhd2* mutants also have short roots, indicating a role of ROS in root elongation as well (Foreman et al., 2003). *latd* mutants also have shorter root hairs than wild-type, in addition to having a short primary root and short root epidermal cells (Liang et al., 2007), similar to the phenotype of *rhd2* mutants.

Studies have shown that ROS molecules regulate plant cell wall composition and function (Liszkay et al., 2004), which could be one mechanism by which ROS regulates cell elongation. Hydroxyl radicals, produced by cell wall peroxidases from  $O_2^{\cdot-}$  or  $H_2O_2$ , are highly reactive and can cleave cell wall polysaccharides, which can cause cell wall loosening and

promote elongation (Fry, 1998; Liskay et al., 2004). On the other hand, peroxidases can also use  $H_2O_2$  as an oxidative substrate to cross-link phenolic monomers and lead to lignin polymerization and restriction to cell elongation (Passardi et al., 2005). Lignin is the second most abundant cell wall polymer after cellulose, and it is crucial for cell wall integrity and stiffness in the vasculature, as well as plant defense responses (Boerjan et al., 2003). Reduced cellulose biosynthesis and cell wall damage caused by environmental stresses can lead to increased lignin deposition, and this process requires and is regulated by ROS production (Caño-Delgado et al., 2003; Hamann et al., 2009; Denness et al., 2011). Ectopic lignin deposition is often associated with plant growth defects, such as altered cell shape, dwarf plant size, or even premature death (Newman et al., 2004; Rogers et al., 2005). Moreover, besides lignin, another specialized cell wall polysaccharide, callose, has also been shown to correlate with ROS (Benitez-Alfonso et al., 2009; Benitez-Alfonso and Jackson, 2009). Callose is enriched at the site of plasmodesmata, a specialized type of channels that mediates cellular transport and cell-to-cell communications, thus plays important role in regulating plant development and responses to environmental stresses (Benitez-Alfonso et al., 2011; Sager and Lee, 2014). The Arabidopsis *gfp arrested trafficking 1* (*gat1*) mutants, which are disrupted in the *GAT1* gene encoding a plastid thioredoxin, have ectopic accumulation of callose and  $H_2O_2$  (Benitez-Alfonso et al., 2009). The *gat1* mutants also have a small root meristem, shorter elongation zone, and growth arrest at about 6 days post germination (Benitez-Alfonso et al., 2009). This finding has provided genetic evidence for the link between cellular ROS homeostasis and callose-mediated cellular trafficking and meristem maintenance (Benitez-Alfonso et al., 2009; Benitez-Alfonso et al., 2011). Since *latd* mutant roots have increased ROS levels and cell elongation defects, as well as altered expression levels of *MtRboh* and cell wall peroxidase genes, whether these changes in *latd* root correlate with lignin and callose deposition is an interesting area to explore.

Since these processes are occurring in the cell wall, they are directly linked to the sensing of signals from outside of the cell, such as abiotic and biotic stresses as well as hormone signaling. Studies examining proteins that can interact with and regulate NADPH oxidases have revealed some answers to the missing link between ROS-regulated growth and perception of extracellular signals. ROS production is under the regulation of RAC/ROP GTPases (Nibau et al., 2006). Interestingly, overexpressing a RAC/ROP GTPase in Arabidopsis, *AtROP2*, leads to increased ROS levels; however, overexpressing *AtROP2* in the *rhd2* mutant background did not exhibit the same effect on increasing ROS levels, or rescuing the root hair phenotype (Jones et al., 2002; Jones et al., 2007). This suggests that the ROP GTPase *AtROP2* function is acting upstream of NADPH oxidase *AtRbohC* in regulating ROS production. In rice, *OsRAC1* can stimulate a transient ROS burst that leads to pathogen resistance, and it also activates the enzyme required for lignin biosynthesis, providing evidence for RAC/ROP GTPase functioning in cell wall modification (Ono et al., 2001; Kawasaki et al., 2006). The activation of RAC/ROP GTPases are controlled by a family of guanine nucleotide exchange factors (GEFs), the ROPGEFs (Berken et al., 2005), and a Receptor-Like Kinase *FERONIA* has been recently shown to interact with *AtROPGEF1* in the regulation of root hair growth (Duan et al., 2010). *FERONIA* has an extracellular domain that can sense extracellular signals and turn on a downstream signaling cascade (Escobar-Restrepo et al., 2007). *FERONIA*, *ROPGEF1*, and *ROP11* all interact with *ABI2*, a member of the Protein Phosphatase 2C family that functions as ABA signaling inhibitors, and this interaction can stimulate the phosphatase activity of *ABI2*, suggesting the *FERONIA*-mediated signaling cascade also functions as a negative regulator of ABA signaling (Yu et al., 2012). These results provide evidence that ABA may trigger downstream ROS signaling and growth responses via a RLK-ROPGEF-RAC/ROP GTPases pathway. However, whether ROP GTPases in *M. truncatula* play a similar role in regulating cell elongation and cross-talk with ABA or other hormonal signaling, is unknown.

Moreover, ROS can also regulate growth by interacting with the cytoskeleton. ROS signaling mediated by RAC/ROP GTPases plays an important role in regulating tip growth in both root hairs and pollen tubes, processes highly associated with the functioning of the actin cytoskeleton (Nibau et al., 2006). During plant defense responses, H<sub>2</sub>O<sub>2</sub> causes cortical microtubule depolarization in Arabidopsis leaves, in an AtRbohD and AtRbohF-dependent manner, and this triggers downstream signaling events to turn on defense gene expression (Yao et al., 2011). ROPGEF4 and ROP Guanosine Triphosphatase (GTPase)-Activating Protein 3 (ROPGAP3) mediate local activation of ROP GTPase ROP11 in xylem cells to initiate a distinct pattern of secondary cell wall differentiation, via inducing local disassembly of microtubules (Oda and Fukuda, 2012). Cortical microtubules can in turn eliminate active ROP11 from the plasma membrane, and these two mutual elimination events regulate the production of the pitted pattern in xylem cells (Oda and Fukuda, 2012). *latd* mutant roots have altered epidermal cell shape, in addition to increased ROS levels (Chapter 2). Whether the increased ROS levels may cause changes in cytoskeleton in *latd* roots, and whether this could contribute to *latd* growth defects, has not been examined at all.

Here we explore the link between ROS, the cell wall and cell elongation as well as cell shape, by examining the expression of several cell wall-associated genes, as well as genes encoding ROP GTPases in *M. truncatula*. We also examined lignin and callose deposition, as well as the organization of cortical microtubules in *latd* roots. We found that *latd* roots have altered expression of a subset of cell wall genes, and slightly but significantly increased expression of MtROP6 and MtROP9, suggesting a possibility that up-regulated ROP GTPase expression may lead to more ROS production in *latd*. Also, young *latd* roots have ectopic deposition of lignin, which might be the reason why *latd* root epidermal cells are shorter. We also



observed ectopic callose deposition in young *latd* roots. Finally, we showed that the cortical microtubule organization is normal in young *latd* roots, but becomes disorganized at an older developmental stage, when the *latd* root meristem arrests. This observation suggests that the microtubule organization defect in *latd* is dependent on the progression of root meristem arrest at later developmental stage, and whether this is a direct cause of shorter cells in *latd* and whether it is associated with increased ROS levels requires closer examination.

## RESULTS AND DISCUSSION

### Cell Wall-Associated Gene Expression Is Altered in *latd* Mutant Roots

*latd* mutant roots exhibit severe growth defects: the primary root is shorter than that of wild-type and eventually stops growing at about 2 weeks post germination (Bright et al., 2005). *latd* lateral roots arrest after they emerge (Bright et al., 2005; Liang et al., 2007). We have recently shown that young *latd* mutant roots have shorter epidermal cells and increased ROS levels (see Chapter 2) (Zhang et al., 2014). Since at this age *latd* mutants have a wild-type looking meristem, the reason why *latd* cells are shorter is possibly not an effect from a dying root meristem. ROS have been shown to play a role in cell wall modifications. H<sub>2</sub>O<sub>2</sub> can cause cell wall stiffening via lignin, while HO<sup>•</sup> can result in cell wall loosening, and these two opposite processes are mediated by peroxidases. We have shown that *latd* mutant roots have slightly higher expression level of a putative cell wall-localized peroxidase in Chapter 2 (Zhang et al., 2014). These observations have lead us to ask whether the cell wall in *latd* roots is altered, and whether the changes in the cell wall may lead to elongation arrest in young *latd* roots. To test this possibility, I checked the expression of genes that encode several cell wall-associated enzymes, *MtXYLOGLUCAN ENDOTRANSGLYCOSILASE* (*MtXET*, Medtr4g126920, TC106155),

MtCELLULOSE SYNTHASE (MtCES, Medtr2g087900, closest homolog in Arabidopsis AtCSLB4), and Mt $\alpha$ -EXPANSIN (Medtr7g010300, TC96054), all of which have been identified in *M. truncatula* (Gimeno-Gilles et al., 2009), in 7-day old *latd* root tips (3 mm). I found that *XET* expression is induced in *latd* root tips (Fig. 3). XETs, which belong to a large family of XYLOGLYCAN ENDOTRANSGLYCOSILASE/HYDROXYLASE (*XTH*), act in the primary cell wall; they can cleave xyloglucan and reform bonds at the non-reducing ends of the xyloglucan available in the primary cell wall (Maris et al., 2009). High XET action has been shown to be present in the growing tip of root hairs and in the root elongation zone of Arabidopsis (Vissenberg et al., 2001; Vissenberg et al., 2005). In growing maize leaves, high XET activity was observed to correlate with the activity of elongation rate (Palmer and Davies, 1996). The different subcellular localizations of XET action depend on different cell types: it either exhibits a fibrillar localization in the epidermal cells in Arabidopsis and in the tobacco root elongation zone and is associated with cellulose microfibrils, or it exhibits a uniform pattern, as seen in the side walls of Arabidopsis root hair cells (Vissenberg et al., 2005). XETs are considered to cause cell wall-loosening, although they can also cause cell wall stiffening by rejoining the new xyloglucans in the primary cell wall (Vissenberg et al., 2005). The explanation of XET activity or action observed in non-growing tissue might be that cell wall crosslinking or lignification can override the cell wall loosening effect from the XETs (Vissenberg et al., 2005). Alternatively, XETs may mainly regulate cell wall restructuring during and even after cell elongation (Vissenberg et al., 2005). This speculation may explain why Mt*XET* expression is higher in *latd* mutant root tips. Interestingly, when I examined the expression of Mt*XET* in 7-day old whole root tissue, *latd* mutants did not show any difference in its expression (Fig. 3). This observation is consistent with the existing findings that the root elongation zone may be the main action site of XETs in regulating cell elongation.

As mentioned above, the subcellular localization of XET action is associated with cellulose microfibrils (Vissenberg et al., 2005). Treatment with an inhibitor of cellulose deposition, as well as using a cellulose synthase mutant, resulted in greatly decreased XET action in the root elongation zone (Fagard et al., 2000; Vissenberg et al., 2005). Another study also showed that *AtXET21* is associated with the thickness of the cell wall and with cellulose deposition; disruption in this gene leads to a short root and short root hairs, abnormal root epidermal cell shape, and dwarf stature (Liu et al., 2007). I wondered if the altered *MtXET* expression in *latd* mutant roots is related to altered cellulose deposition. A cellulose synthase in *M. truncatula*, *MtCELLULOSE SYNTHASE* (*MtCES*) (Medtr2g087900, TC104491), did not exhibit any significant difference at expression level in the root tips, although in the whole *latd* root tissue, it is down-regulated more than 3-fold (Fig. 3). It is possible that this *MtCES* functions in the mature region of the roots, although it is hard to conclude whether *latd* mutant roots have defects in cellulose deposition without checking more cellulose synthase gene expression or quantifying cellulose in the cell wall. It is the case also for the *Mt $\alpha$ -EXPANSIN*, which belongs to a multigene family (Li et al., 2002), and function in cell wall loosening by unlocking the network of polysaccharides and permitting turgor-driven cell enlargement (Cosgrove, 2000).  *$\alpha$ -EXPANSIN* expression did not exhibit any significant difference in *latd* roots, but since there are many  *$\alpha$ -EXPANSIN* genes in the same family, I cannot conclude that  *$\alpha$ -EXPANSIN* expression is unaltered in *latd* roots.

### **ABA Regulates Cell Wall-Associated Gene Expression in *latd* Mutant Roots**

ABA inhibits primary root elongation in *Arabidopsis* and *M. truncatula* (Cheng et al., 2002; Liang and Harris, 2005; Liang et al., 2007). We have shown in Chapter 2 that ABA inhibits cell elongation in wild-type primary roots, but stimulates elongation in *latd* roots (Zhang et al.,

2014). We also showed that ABA increases *latd* root cell length, at least in part, by decreasing ROS production (Zhang et al., 2014). We wondered whether ABA also affects cell wall gene expression in *latd* roots. The Arabidopsis mutant *abscisic acid-insensitive 8 (abi8)*, insensitive to ABA for seed germination, stomatal closure, and ABA-responsive gene expression, also exhibits cell wall defects in cellulose deposition (Cheng et al., 2000; Pagant et al., 2002; Brocard-Gifford et al., 2004). This suggests that ABA may play an important function in the regulation of the cell wall, so that insensitivity to endogenous ABA would lead to cell wall defects. I wondered whether ABA has any effect on cell wall-related gene expression. We found that MtXET expression is up-regulated by 10  $\mu$ M ABA in both wild-type and *latd* root tips, but not in whole root tissue (Fig. 3). In contrast, MtCES expression is strongly repressed by ABA, both in the root tips and the whole roots (Fig. 3), while Mt $\alpha$ -EXPANSIN expression was not altered by ABA in wild-type or *latd* root tips (Fig. 3). In contrast, ABA can up-regulate Mt $\alpha$ -EXPANSIN expression in whole roots of both wild type and *latd* mutants (Fig. 3), suggesting that ABA may regulate Mt $\alpha$ -EXPANSIN differently in the root tips, where cells are dividing and expanding, than in the mature region of the roots where cells are no longer growing and differentiating. The expression profile of MtCES and Mt $\alpha$ -EXPANSIN correlate well with our finding that ABA reduces cell elongation in *M. truncatula* primary roots shown in Chapter 2 (Zhang et al., 2014). Our results here suggest that this effect of ABA on cell elongation may be due to down-regulation of genes involved in cell wall biosynthesis and cell wall loosening, which are required for cell elongation.

### **Ectopic Lignin and Callose Deposition in *latd* Mutant Roots**

ROS molecules can inhibit growth by cross-linking cell wall polymers, which leads to cell wall stiffening and growth restriction (Gapper and Dolan, 2006). H<sub>2</sub>O<sub>2</sub> has been shown to help cross-link cell wall phenolic polymers, such as lignin, via cell wall peroxidases (Ros-Barceló

et al., 2002; Passardi et al., 2005). There is also evidence that  $H_2O_2$  production stimulates secondary wall cellulose synthesis and the differentiation of the secondary cell wall (Potikha et al., 1999). Since *latd* roots have an increased level of ROS and cell elongation defects (Zhang et al., 2014), and ROS stimulates lignin formation, we wondered if the *latd* mutant has increased root lignin deposition. To test this, I used a standard phloroglucinol staining method to examine lignin localization and accumulation in *latd* roots (Jensen, 1962). I found that in young wild-type and *latd* mutant roots (7-days old), *latd* roots have ectopic lignin deposition, beginning approximately 5 mm from the root tips, while in wild-type roots, no significant level of lignin can be detected at this location (Fig. 4). The increased lignin staining is also present further long the primary roots of *latd* mutant (Fig. 4B). Even in older regions of the root, where lignin staining is also present in wild-type roots, *latd* mutants still have stronger lignin staining (Fig. 4C). Interestingly, *latd* hypocotyls also exhibit increased lignin staining, as compared to wild-type plants (Fig. 4D). These observations suggest that *latd* mutants do exhibit increased lignin deposition in both roots and hypocotyls, and, more importantly, *latd* roots have ectopic lignin deposition in the very young region of the roots, where cells are undergoing elongation and differentiation. Whether this ectopic lignin deposition in *latd* roots is a result of increased ROS levels, and whether the lignin increase is the actual cause of decreased cell elongation in *latd* mutants, would be very interesting to investigate.

Ectopic callose deposition can lead to defects in cell-to-cell trafficking via plasmodesmata, and its production is stimulated by increased ROS levels,  $H_2O_2$  specifically (Benitez-Alfonso and Jackson, 2009; Benitez-Alfonso et al., 2011). Since *latd* roots accumulate more ROS than wild-type roots, we asked whether this increased ROS level could lead to ectopic callose deposition. To test this, I used aniline blue to stain callose in *latd* mutant roots. I found that callose staining in mature roots is mostly observed in the vasculature, and there is increased

callose staining in the mature region of *latd* roots, compared to wild type (Fig. 5). This indicates that *latd* mutant roots do have increased callose deposition, but if *latd* mutants have any defect in intercellular trafficking, and if this contributes to the growth defects in *latd*, needs to be determined.

### **ROP GTPases Exhibit Altered Expression Levels in *latd* Mutant Roots**

ROP GTPases not only play an important role in regulating ROS-mediated cell elongation in root hairs and pollen tube growth, but also affect cell wall composition (Nibau et al., 2006). Since *latd* mutant roots have defects in root elongation, and have elevated ROS levels, as well as ectopic lignin deposition in the cell wall, we wondered whether the expression of ROP GTPases could be altered. We checked the expression levels of three ROP GTPase-encoding genes, *MtROP5*, *MtROP6* and *MtROP9*, and we found that *MtROP6* and *MtROP9* have slightly, but significantly, increased expression levels in *latd* roots (Fig. 6). Interestingly, *MtROP5* expression is decreased in *latd* plants both when grown on BNM and in the presence of ABA (Fig. 6). Whether these ROP GTPases actually contribute to the increased ROS levels in *latd* roots would be an interesting area to explore, and looking at how ABA may regulate these ROP GTPases would provide more insight into the way in which ABA regulates ROS levels during root growth.

### **Defects in Microtubule Organization in *latd* Mutants Are Developmental Age Associated**

Cortical microtubule organization is closely correlated with cell elongation and cell shape. It has been found in many plant species that in the root elongation zone, microtubule orientation is transverse to the direction of cell elongation (Blancaflor, 2000; Ehrhardt and Shaw,

2006). Disruption of this organization, either using drugs or mutants can inhibit cell elongation and induce radial expansion of the cell, thus causing changes in cell shape (Blancaflor, 2000; Wiedemeier et al., 2002). Because *latd* mutant roots have shorter cells and altered cell shape, we wondered whether the microtubule cytoskeleton is affected in *latd* roots. We used antibodies against alpha tubulin by immunolabeling (Dyachok et al., 2009), in whole roots of 4-day old wild-type and *latd* plants. At this early developmental stage, *latd* mutant roots grow at a similar rate as wild-type, although *latd* roots were already shorter (Bright et al., 2005). We found that in 4-day old *latd* roots, microtubule organization in the root meristem appears wild-type, and is oriented mostly in a transverse direction (Fig. 7). We also observed normal patterns of cell files in the *latd* root meristem, as well as cells undergoing cell division (Fig. 7, arrowheads). In the elongation zone of *latd* roots, the transverse orientation of microtubules is also very similar to that of wild-type roots (Fig. 7). Once cells have completed elongation, microtubule orientation changes from a transverse direction to one that is more parallel to the direction of elongation, and this dynamic reorganization is also seen in *latd* roots, in a very similar way to wild-type roots (Fig. 7). Therefore, we can conclude that at an early developmental stage, *latd* roots do not have any defects in the orientation and the dynamics of microtubule organization, and the fact that young *latd* roots are shorter is probably not caused by changes in microtubule organization.

Root growth defects, as well as cell elongation defects in *latd* mutant roots, become more severe as development progresses (Bright et al., 2005; Zhang et al., 2014). The *latd* root growth rate gradually comes to a full stop in 2 weeks to 3 weeks post germination, and at this time, the *latd* root meristem becomes very disorganized (Bright et al., 2005; Liang et al., 2007). When we examined microtubule organization at this later developmental stage, in 3-week old *latd* mutants, we found that the microtubule orientation is clearly disorganized; the transverse orientation of microtubules in the elongation zone is absent, and instead, most cells have microtubules parallel

to their growth direction. The *latd* root apical meristem at this time no longer has obvious cell files, cells are unorganized, scattered and cell shapes have become irregular, as previously reported (Bright et al., 2005). The microtubule pattern reflects the disorganization of the meristem at this stage (Fig. 8). It is not clear whether the microtubule changes happen first, then lead to growth defects in *latd* roots at an older age, or whether the growth defects cause the reorganization of microtubules.

In summary, I have found that in young *latd* mutant roots, the expression of several cell wall-associated genes that are involved in cell wall loosening and synthesis is altered, and that ABA regulates the expression of several of these genes in a tissue-specific manner. Lignin and callose accumulate ectopically in the *latd* root cell wall, although the disorganization of the microtubule cytoskeleton seems to be a result of progressive developmental defects in old *latd* roots. In addition, I found that two ROP GTPases have increased expression levels in *latd* roots, which provides a possible reason for why *latd* roots have increased ROS levels as well as ectopic lignin deposition.



## **APPENDIX III. FUNCTIONAL CHARACTERIZATION OF TRANSCRIPTION FACTORS DOWNSTREAM OF ABA AND MTLATD/NIP SIGNALING PATHWAY**

### **INTRODUCTION**

ABA signaling transduction involves transcriptional reprogramming via transcription factors (TFs). In *Arabidopsis*, many TFs have been identified as downstream transcriptional regulators of ABA signaling, which mediates changes in transcriptional levels of genes functioning in stress tolerance (Finkelstein, 2013). In the model legume *M. truncatula*, little is known about TFs that function in ABA signaling, and very few TFs have been identified to be regulators downstream of ABA in root development.

In Chapter 3, we used TF profiling to identify TFs whose expression levels are regulated by ABA or the nitrate transporter MtLATD/NIP. MtLATD/NIP also mediates ABA signaling and is required for root and nodule meristem function as well as changes in ROS-related gene expression (Zhang et al., 2014); however, the mechanism by which MtLATD/NIP regulates gene expression, is not clear. We have identified TFs that are regulated by both MtLATD/NIP and ABA in Chapter 3, and here I will report on recent progress to functionally characterize two TFs, a GATA Zinc finger (ZF) protein MtGATA9 (Accession No. Medtr3g109760), and a NAC domain-containing protein MtNAC104 (Accession No. Medtr8g093790).

### **RESULTS AND DISCUSSION**

#### **Transcriptional Regulation of MtGATA9 and MtNAC104 by MtLATD/NIP, ABA and Nitrate**

From TF profiling results, we found that *MtGATA9* expression is regulated by both MtLATD/NIP and ABA in 4-day old roots (Chapter 3, Supplemental Table S4) and this chapter, Fig. 9). The expression of *MtGATA9* is slightly up-regulated in *latd* mutant roots, and it can be induced more than 3-fold by continuous ABA treatment in both wild-type and *latd* roots (Fig. 9). *MtNAC104* expression is up-regulated by ABA, both in wild-type and in *latd* mutant roots, in a very similar manner; there is no difference in expression between untreated wild-type and *latd* roots (Fig. 9). Since MtLATD/NIP is a nitrate transporter, I also examined the expression levels of these two TFs in response to a 3-day treatment with 10 mM KNO<sub>3</sub>. Interestingly, 10 mM KNO<sub>3</sub> can down-regulate the expression of *MtGATA9* in wild-type roots, but this regulation is absent in *latd* mutant roots (Fig. 10). Curiously, a 3-day ABA treatment done in parallel did not lead to an induction of *MtGATA9* expression as seen in the TF profiling (Fig. 9), but was instead indistinguishable from wild-type (Fig. 10) suggesting that *MtGATA9* either responds differently to ABA after continuous exposure, or else the difference is due to the plant developmental stage, since this 3-day treatment was done on 1-week old plants, making them 10-days old when harvested, much older than the plants used in the TF profiling. Another interesting observation is that in 10-day old plant roots, *MtGATA9* expression in *latd* is significantly lower than that in wild type (Fig. 10), rather than slightly higher, as in the TF profiling (Fig. 9) suggesting that this *MtGATA9* may be responsive to the progressive loss of the meristem in *latd* roots, as they get older. In contrast, *MtNAC104* expression in response to a 3-day ABA treatment (Fig. 10) is similar to what was seen for the TF profiling (Fig. 9), except that ABA stimulation of expression in *latd* mutants is now significant. We found that *MtNAC104* expression is not responsive to a 3-day 10 mM KNO<sub>3</sub> treatment in wild-type roots, but it is slightly up-regulated in *latd* mutant roots.

*MtGATA9* is predicted to be a TF of the GATA ZF family, which contains a DNA binding domain with a type IV ZF in the form CX<sub>2</sub>CX<sub>17-20</sub>CX<sub>2</sub>C followed by a highly basic

region at its C-terminus (Reyes et al., 2004). MtNAC104 is predicted to be a TF of the NAC family that contains a NAC domain. Based on the Medicago Gene Expression Atlas, both MtGATA9 and MtNAC104 are highly expressed in the root tips (3 mm) (Benedito et al., 2008), just as MtLATD/NIP is (Yendrek et al., 2010). Thus, it will be interesting to look at the expression of MtGATA9 and MtNAC104 in *latd* mutant root tips.

### **Overexpression of MtGATA9 and MtNAC104**

Since MtGATA9 and MtNAC104 are under the regulation of ABA and MtLATD/NIP, we wondered whether they could be playing a role in root or nodule development. To test this, I overexpressed MtGATA9 and MtNAC104 in *M. truncatula* wild-type and *latd* mutant roots using *Agrobacterium rhizogenes* transformation (Boisson-Dernier et al., 2001; Limpens et al., 2004). Composite wild-type and *latd* plants carrying these constructs were grown in the greenhouse for 4 weeks, and this process was repeated twice. We found no significant difference in root or shoot dry weight between composite plants carrying the Mt GATA9 overexpression construct or the empty vector control in either wild-type or *latd* mutants. However, root dry weight was increased in *latd* roots overexpressing MtNAC104 (Fig. 11A and B). MtNAC104 overexpression was confirmed functional by examining MtNAC104 expression in the transformed roots, which was dramatically increased compared to those transformed with empty vector (Fig.11D). Also, nodule number and nodule density (nodule number per cm primary root) at 2 weeks post-inoculation increased in both MtGATA9 and MtNAC104 overexpression lines, in the wild-type background (Fig. 12A and B). *latd* mutants did not form any large nodules, as previously reported (Bright et al., 2005). However, these results did not seem to be consistent, since the results were not reproduced in the second biological replicate (Fig. 11C). This is possibly due to the fact that the control in the second replicate overexpresses the GUS protein, instead of using an empty vector;

but GUS overexpression has been used in many studies as controls (Gonzalez-Rizzo et al., 2006; De Zélicourt et al., 2012). Also, composite plants were over 1 month old growing in the greenhouse, where it is difficult to maintain consistent growth conditions. An alternative method would be to transfer composite plants to growth pouches, where root growth can be easily followed. This modified method was used in Chapter 2 and the results are promising and reproducible, and does not require too long of a process. Future studies should be carried out using this modified protocol.

In summary, *MtNAC9* and *MtNAC104* are potentially interesting downstream TFs that may play a role in MtLATD/NIP and ABA signaling in the regulation of root development. To further investigate the exact role of these two TFs, repeating the overexpression analysis with a modified method is necessary. Also, reducing their expression using RNA interference, as well as promoter analysis, will provide important information on the functions of these TFs as well as their spatial pattern of expression. Moreover, since *MtNAC104* is specifically induced by drought stress (Benedito et al., 2008), it will be interesting to examine whether *MtNAC104* plays a role in mediating drought stress responses and tolerance via ABA.

## APPENDIX IV. CHARACTERIZATION OF A ROOT HAIR MUTANT OF *MEDICAGO TRUNCATULA*

### INTRODUCTION

Legumes form a specialized root organ, nodules, via a symbiotic interaction with nitrogen-fixing rhizobia. Nodule formation is a highly coordinated process consisting of bacterial infection initiating in the epidermis and cell division in the cortex, many cell layers below (Oldroyd and Downie, 2008). The root epidermis is the first location where legume roots and rhizobia come in contact in most forms of the legume-rhizobia symbiosis, and it is the first regulatory site that could influence where, when, and how many nodules will be formed (Oldroyd and Downie, 2008). Nodulation is activated by the perception of plant-secreted flavonoids by the rhizobia, and then rhizobia will produce signaling molecules, Nodulation factor (Nod factor) that can be recognized by the plant. Nod factor perception then triggers the downstream symbiosis signaling pathway. Nod factor is perceived by LysM receptor-like kinases, which contain an extracellular LysM domain (Madsen et al., 2003; Radutoiu et al., 2003). Nod factor perception by the receptor-like kinase complex NFP1-NFR5 in *L. japonicas* and LYK3-DMI2 in *M. truncatula*, then leads to calcium oscillations, termed calcium spiking, in the nucleus via two cation channels located on the nuclear membrane, CASTOR AND POLLUX in *L. japonicas* and a single inner-membrane-localized channel DMI1 in *M. truncatula* (Oldroyd, 2013). Calcium spiking is then perceived and transduced by CCaMK/DMI3 in *M. truncatula*, and transcription factors NSP2, NSP1 and ERN (Oldroyd, 2013). The signaling transduction then leads to the induction of *EARLY NODULIN 11* (*MtENOD11*) gene expression (Journet et al., 1994; Journet et al., 2001).

Root hair cells in the epidermis play an important role in the bacterial infection process during nodulation. In the root epidermis, rhizobia attaching to the root hair cells is the first step of

rhizobia associating to the plant (Oldroyd and Downie, 2008). Nod factor perception induces root hair deformation, a transient interruption of root hair tip growth, which leads to root hair swelling and branching; and when growth resumes, it results in root hair bending or curling that eventually entrap the rhizobia in the root hair curl and forms an infection pocket (Esseling et al., 2003; Oldroyd et al., 2011). The entrapped rhizobia will continue dividing and form colonies called infection foci, which are the starting points of root hair infection thread (IT) development (Oldroyd et al., 2011). ITs continue to elongate, passing several cortical cell layers, and eventually reach the developing nodule primordia, where bacteria in the symbiosomes are released and terminally differentiated into bacteroids that can fix nitrogen.

Nod factor signaling and the process of bacterial infection are well demonstrated, however, genetic regulatory components in the host plant still have many unanswered questions. Root hair cells are the Nod factor perception sites and lead to Nod factor signaling and bacterial infection in later steps, however, is root hair required for establishing symbiosis in *M. truncatula*? Is there alternative way for this process to occur? Will the absence of normal root hairs affect nodulation events in epidermal cell layer, as well as that in the root cortex? Studies done in *L. japonicas* root hair mutant *root hairless 1* (*Ljrh1*) have revealed an interesting answer: the presence of root hairs is not required for the activation of cortical program, however, it is essential for the establishment and progression of infection in the epidermis (Karas et al., 2005). Mutants defective in root hair development have not been characterized in *M. truncatula*, but several mutants have been identified of having defects in root hair responses during nodulation, including the *hair curling* (*hcl*) mutants, which are defective in forming root hair curls, microtubular reorientation and IT in response to rhizobia, as well as decreased cortical cell division (Catoira et al., 2001).

In a previous genetic screen in our lab for mutants defective in nodulation, a mutant with altered root hair development was isolated. Here I will describe the genetic characterization and the observations of mutant phenotypes.

## **RESULTS AND DISCUSSION**

### **Phenotypic characterization of a Root Hair Mutant of *M. truncatula***

In a screen of fast neutron bombarded *M. truncatula* cv. Jemalong A17 population for mutants defective in early plant development as well as nodulation, a mutant with a root hair phenotype was isolated (David Mitchell and Jeanne M. Harris, unpublished data). The initial phenotypic characterization was done on the unbackcrossed M3 generation. We found that the root hairs are severely shortened in these mutants, as compared to wild type (Fig. 13A and B). A curly hypocotyl can also be observed in some mutants (Fig. 13C and D). Because of the short root hair phenotype, we named this mutant *duan fa* (*dfa*) (short hair in Chinese).

Because root hairs play an important role in infection during the development of symbiotic nodules, we examined nodulation in these mutants. We found that the mutants do form pink nodules, indicating the root hair phenotype did not abolish nodulation or subsequent nodule development. We also observed root hair deformation and curling in response to *Rhizobium* inoculation (Fig. 13E and F). However, the nodule number is decreased in *dfa* mutants (Fig. 13G).

### **Genetic Characterization of the *dfa* mutant**

The root hair mutant was backcrossed to the wild-type A17 line once, using the mutant as male and A17 as female. The F2 progeny resulting from the F1 plants were collected, and scored for the wild-type and mutant root hair phenotype. We found that the short root hair phenotype segregated in approximately a 3:1 ratio (200:66,  $\chi^2 = 0.160$ ,  $P = 0.9$ ). Therefore, the short root hair phenotype is recessive.

Since we occasionally observed the curly hypocotyl phenotype in the segregating F2 population (Fig. 13H and I), we also wondered if the short root hair phenotype and curly hypocotyl phenotype might cosegregate, indicating that they could be the result of a mutation in a single gene. Again, the F2 progeny from the first backcross was used for cosegregation analysis (Table 5). We found that the root hair phenotype and curly hypocotyl phenotype failed to cosegregate ( $\chi^2 = 10.40$ ,  $P < 0.005$ ), therefore the two mutant phenotypes are not due to a mutation in the same gene.

Then we wondered if the short root hair and curly hypocotyl phenotypes might be the result of mutations in two unlinked genes. We observed that ratio of plants with the wild-type phenotype: short root hair: curly hypocotyl: short root hair plus curly hypocotyl phenotype is 59:10:12:16, with  $\chi^2 = 22.44$ ,  $P < 0.005$ . Thus, we rejected the hypothesis that the short root hair phenotype and curly hypocotyl phenotype are due to two separate mutations in two unlinked genes. Since both hypotheses are rejected, i.e., the root hair and hypocotyl phenotype are not due to mutation either in a single gene or in two unlinked genes, the likeliest alternative explanation is that this pattern of phenotypes is due to two linked genes.

Another interesting observation is that after the backcross, I selected a plant with both the root hair and curly hypocotyl phenotype from the F3 population, and after I harvested seeds from



this plant, all its progeny have the curly hypocotyl phenotype (Fig. 13H and I) as well as a more severe root hair phenotype, with no root hair development observed at all (Fig. 13 J and K). Since I demonstrated that these two phenotypes are mostly likely due to mutations in two linked genes, we will need to pick an F2 progeny that has only the root hair phenotype or only the curly hypocotyl phenotype, and backcross to wild-type plants again, in order to separate these two mutations.

In summary, I have started the initial genetic and phenotypic characterization of a root hair mutant *dfa* of *M. truncatula*, which exhibits short root hair and decreased nodule number. I have determined that the line contains a linked mutation that affects hypocotyl curling. Future study will be needed to to separate the linked hypocotyl phenotype from the root hair phenotype and complete the genetic analysis of the genes.

## MATERIALS AND METHODS

### Plant Growth Conditions

*M. truncatula* seeds were scarified with concentrated sulfuric acid for 10 minutes, rinsed 6 times with sterile water, and sterilized in 30% Clorox, before imbibing for 5 to 6 hours, shaking at room temperature. Seeds were cold-treated for at least 24 hours before germinating in a moistened, sealed petri plate overnight in the dark. The A17 line was used as the wild-type control in all experiments. Seedlings were grown on 25 cm x 25 cm petri dishes (Nunc, <http://www.nuncbrand.com/>) or in growth pouches (<http://www.mega-international.com/>) containing buffered nodulation medium (BNM) at pH 6.5 (Ehrhardt et al., 1992) and placed

vertically in an MTR30 Conviron growth chamber at 20°C, 50% humidity, 16 h light/8 h dark cycle with an intensity of 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ . Growth pouches were placed in a sealed Styrofoam box with a clear plastic dome as a lid. For ABA or KNO<sub>3</sub> treatment, ( $\pm$ )-ABA (A1049; Sigma, <http://www.sigmaaldrich.com/>) or 1 M KNO<sub>3</sub> stock solution were added to the medium after autoclaving to reach specified concentrations.

For unstained root cross sections, roots were cut into 1 cm segments, embedded in 3% (w/v) agarose and sectioned on a Lancer Vibratome Series 1000. All sections were 90  $\mu\text{m}$  thick. Images were taken using an Olympus microscope.

### **Lignin and Callose Staining, Microtubule Cytoskeleton Immunolabeling and Confocal Microscopy**

For lignin staining, whole roots were incubated in saturated phloroglucinol prepared in 20% (w/v) HCl (Jensen, 1962) for 15 minutes, then imaged under a Leica dissecting scope. Staining was repeated twice, with a total of 4 to 5 roots of each genotype.

For callose staining, whole roots were incubated in 0.025% (w/v) aniline blue in 0.1 M sodium phosphate buffer (pH 7.0) for 1 hr, and then washed with fresh buffer 2-3 times. Stained roots were then imaged using a Zeiss confocal microscope under UV illumination. This staining was repeated twice, with a total number of 4 to 5 roots per genotype.

Microtubule cytoskeleton staining was slightly modified from (Dyachok et al., 2009). The first 1 cm root segments containing root tips were harvested and embedded in PME buffer

containing 10% (v/v) DMSO and 3.7% (w/v) formaldehyde, followed by 10 min vacuum infiltration. After incubating for 1.5 hr, tissue was washed with fresh PME buffer for 3 times and longitudinal sections were obtained on a Lancer Vibratome. All sections were 80  $\mu$ m thick. Sections were placed on coverslips then mounted with a thin layer of agar on top to secure their positions. Then 1% cellulase YC in PME buffer was added and washed in 5 min. 1% (v/v) Triton X-100 in PME was added to the specimen on the coverslips and sat for 10 min, then washed thoroughly. Primary monoclonal rat antibody anti-tubulin was added on the cover slips, which then were kept in a petri dish overnight. The next day, cover slips were washed with fresh PME buffer, then secondary antibody goat anti-rat immunoglobulin G conjugated to fluorescein isothiocyanate (Sigma-Aldrich) was applied. After incubated for 2 hours, sections were washed and mounted with 20% Mowiol mounting media (Calbiochem, La Jolla, Calif., U.S.A.) then placed in the dark overnight. On the third day, images were taken on a Biorad confocal microscope using excitation at 488 nm and emission detected at 500-550 nm. The staining was done on a total of 3 to 5 roots each genotype.

### **Construction of Overexpression Vectors and *Agrobacterium rhizogenes* Transformation**

The coding sequence of Mt*GATA9* and Mt*NAC104* was amplified from wild-type root cDNA using Invitrogen high-fidelity polymerase *Pfx* (primers listed in Table 4). The purified PCR fragments were then cloned into the GATEWAY pENTR Directional TOPO Cloning kit (Invitrogen) following the manufacturer's instructions, and recombined into the destination vector pB7WG2D,1 (VIB Plant Systems Biology, University of Ghent) (Karimi et al., 2002) using Gateway cloning (Invitrogen) to produce the overexpression vectors pCZ22 and pCZ23, containing Mt*GATA9* and Mt*NAC104* respectively. Vectors were transformed into *Agrobacterium rhizogenes* strain Arqua1 (Quandt et al., 1993) and then used to transform both A17 and *latd*

*Medicago truncatula* roots (Limpens et al., 2004). As a control, some plants of each genotype were transformed with a GUS overexpression construct pCZ41. After two weeks, transgenic roots were identified by screening for GFP fluorescence using the blue excitation filter on a Nikon TE200 fluorescence microscope. Non-transformed roots were cut off as were all but one transformed root. Composite plants containing untransformed shoots and a single transformed root were then transferred to pots containing turface and sand (v/v, 1:1) and placed in the greenhouse. Plants were watered with ½x liquid BNM medium every two days. At 4 weeks, plants were harvested from the greenhouse: 3-5 individual transformation lines were harvested in liquid nitrogen and stored at -80°C for RNA extraction; all remaining roots and shoots were dried separately at 60°C for 2 days, and dry weight was recorded.

### **Quantitative RT-PCR**

Total RNA was extracted from whole roots using RNeasy plant mini kits (Qiagen) following the manufacturer's protocol. Each treatment in all experiments was from 40 pooled 3 mm root tips; all experiments were repeated at least three times. RNA was quantified with a nanodrop, followed by DNase treatment (Turbo DNase-free kit, Ambion). RNA was subsequently cleaned and concentrated with Qiagen RNA Cleanup Kit. All RNAs were check for quality and integrity with an Agilent 2100 Bioanalyzer. cDNA was synthesized from one µg of total RNA using the SuperScript III first-strand synthesis system (Life Technologies, Invitrogen). qRT-PCR was performed using either an ABI StepOnePlus or ABI 7900HT Real-time PCR system (Applied Biosystems), using SYBR Green reagent (VWR). Data was collected with SDS 2.2 software (Applied Biosystems). Target gene relative expression was determined by normalizing with the expression of endogenous control UBC9 and/or PDF2 (Kakar et al., 2008). Statistical analysis was performed using one-way ANOVA in SPSS software (Version 20.0.0) on the

relative gene expression from 3 biological replicates. Primers used in qRT-PCR were designed using Primer Express v3.0.1 software and are listed in Table 1-3.

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## TABLES

**Table 1. qRT-PCR primers used in Appendix I.**

Gene	Accession	Reference	Primer Sequence (5'-3')
Mt <i>PLT1</i>	AY506549	(Imin et al., 2007)	F: TTGTTGCATGAATAGATGATTTGAGA
			R: CCTTCTTCAAGATACATGCCAATG
Mt <i>PLT2</i>	AY506450		F: CATCAATGTACCGTGGAGTTACAAG
			R: TCCTGCAACTCTGCCAATCC
Mt <i>WOX5</i>	BF649819		F: GGAATCCAACAACAGAACAAGTTAAA
			R: TGAATTTGATCAGTGCTTGGAGTT
Mt <i>STM</i>	TC99129		F: CAAAGGAAACGGCACTGGAA
			R: CCATGTAATAGTGTGGATGACTTGGA
Mt <i>BBM</i>	AY899909		F: TTGTTGCATGAATAGATGATTTGAGA
			R: CCTTCTTCAAGATACATGCCAATG
Mt <i>SCR</i>	Medtr7g074650		F: TTTCTCAGCAACAGGGAAAGAAC
			R: CCCTTGAAGCTACTGGTAAACGTT
Mt <i>SHR</i>	Medtr4g097080		F: TTGCACTCAATGGCCAACAC
			R: CGAAGGTGAGGCGTATCATCA
Mt <i>CYC-LIN B</i>	AJ621872	(Gimeno-Gilles et al., 2009)	F:AGGGAAC TTCAATTGGTTGGAA
			R:GGCCAGATTTCTTCATACTTCGA
Mt <i>UBC9</i>	Medtr7g116940	(Kakar et al., 2008)	F: GGTTGATTGCTCTTCTCTCCCC
			R: AAGTGATTGCTCGTCCAACCC

**Table 2. qRT-PCR primers used in Appendix II.**

Gene	Accession No.	Reference	Primer Sequence (5'-3')
Mt <i>XET</i>	Medtr4g126920	Designed in this study	F:AGCCCCATTCACTGCTTCAT R:GAGGAACCTGAAGAAGTCCAAACA
Mt <i>CES</i>	Medtr2g087900		F:GGCCAAAACAACCACATCATC R:CATCAATCCAGAGACTCTTGTCAG
Mt $\alpha$ - <i>EXPANSIN</i>	TC96054		F:CTGTGGCCATCAAAGGTTCA R:TTGCCCCCAATTCCTTGAC
Mt <i>ROP5</i>	TC125149		F:CATTCTCGCTTTCTCCCTCATAA R:TGGAATCCACTTTTTGGAAACA
Mt <i>ROP6</i>	TC177831		F:TGCGGCGCCAATCAC R:CTGGAGCACCGATAAGTTTTCTC
Mt <i>ROP9</i>	TC117816		F:TTGTCCAACCTCCAAGGA R:ACCGGACCTTCTTTGCCTTT
Mt <i>UBC9</i>	Medtr7g116940	(Kakar et al., 2008)	F:GGTTGATTGCTCTTCTCTCCCC R:AAGTGATTGCTCGTCCAACCC
MtPDF2	Medtr6g084690		GTGTTTTGCTTCCGCCGTT CCAAATCTTGCTCCCTCATCTG

**Table 3. qRT-PCR primers used in Appendix III.**

Gene	Accession No.	Medicago Gene Expression Atlas	qRT-PCR Primers (5'-3') (Designed in Chapter 3)
Mt <i>GATA9</i>	Medtr3g109760	Mtr.48513.1.S1_ at	F:CGAACTCTCCGTTCCGTATG R:ATTTGGTGCCTTCAAACCAG
Mt <i>NAC10</i> 4	Medtr8g093790	Mtr.15417.1.S1_ at	F:GGAGTGAAAGCAGTGTGAAGC R:CCAAGGATCATGGTGAGACA

**Table 4. Primers used in constructing overexpression vectors in Appendix III.**

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Mt <i>GATA9</i>	CACCATGGGAAGCTCAAGAGTTTT TCC	CTAGATGAGGTGCGTGAAATCG
Mt <i>NAC10</i> 4	CACCATGATGGGGAGTGAAAGCA G	CTAATTAGGTAGGCTTGTTTGATC AAG

**Table 5. Chi-square test**

**Hypothesis 1:** The root hair phenotype and curly hypocotyl phenotypes are due to a mutation in a single gene.

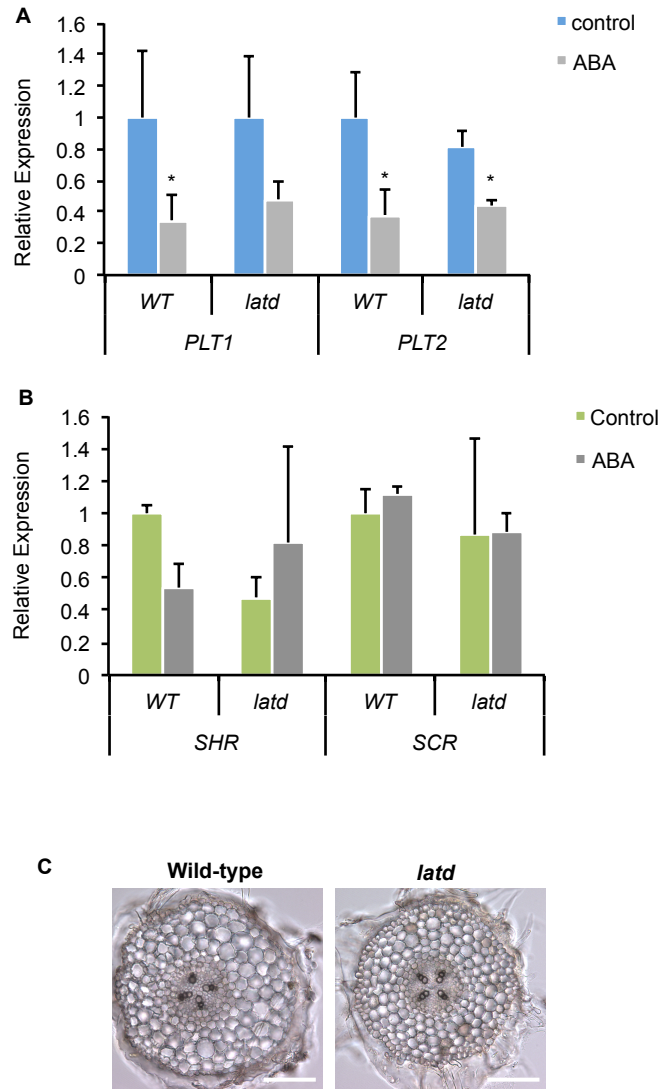
phenotype	observed	expected	(obs-exp) <sup>2</sup> /exp	Degree of freedom	Chi-square	Conclusion
Wild-type	59	72.75	2.5988	1	10.40	Rejected
Short root hair or curly hypocotyl	38	24.25	7.7964			

**Hypothesis 2:** The root hair phenotype and curly hypocotyl phenotypes are due to mutations in two unlinked genes.

phenotype	observed	expected	(obs-exp) <sup>2</sup> /exp	Degree of freedom	Chi-square	Conclusion
Wild-type	59	54.56	0.3609	3	22.44	Rejected
Curly hypocotyl	10	18.19	3.6858			
Short root hair	12	18.19	2.1050			
Curly hypocotyl and curly root hair	16	6.06	16.2893			



## FIGURES



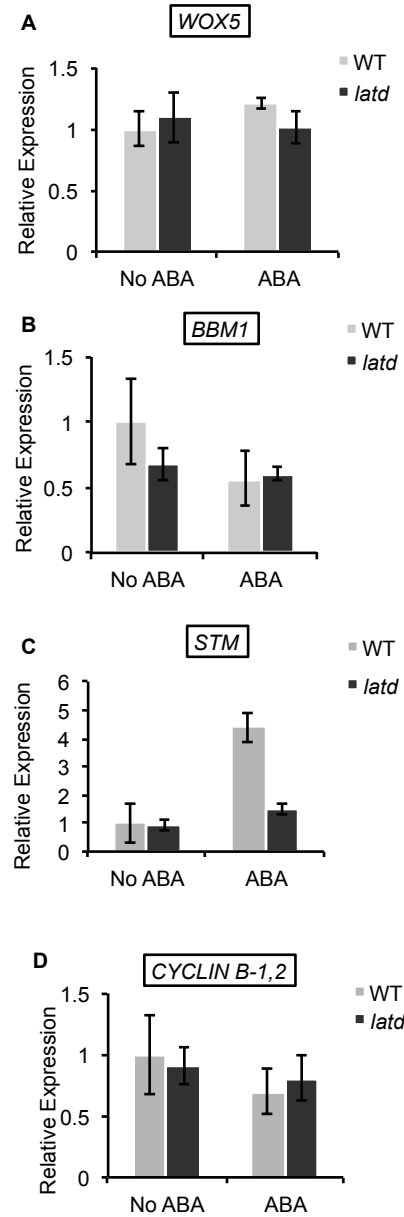
**Figure 1. Root meristem genes have normal expression levels in young *latd* root tips.**

Relative expression of *MtPLT1* and *MtPLT2* (A), *MtSHR* and *MtSCR* (B) is not altered in young *latd* mutant root tips. ABA only down-regulates the expression of *MtPLT1* and *MtPLT2* (A), but not that of *MtSHR* and *MtSCR* in root tips.

Root tips (3 mm) from seven-day old plants grown on BNM plates with or without 10  $\mu$ M ABA were harvested for qRT-PCR analysis. Graphs represent the average from three biological

replicates for *MtPLT1* and *MtPLT2*, two biological replicates for *MtSHR* and *MtSCR*, with each gene normalized to *MtUBC9* as endogenous control. Error bars represent standard error. Asterisks indicates a statistically significant difference between treatments in the same genotype, using one-way ANOVA test, with a p-value <0.05. qRT-PCR primers are listed in Table 1.

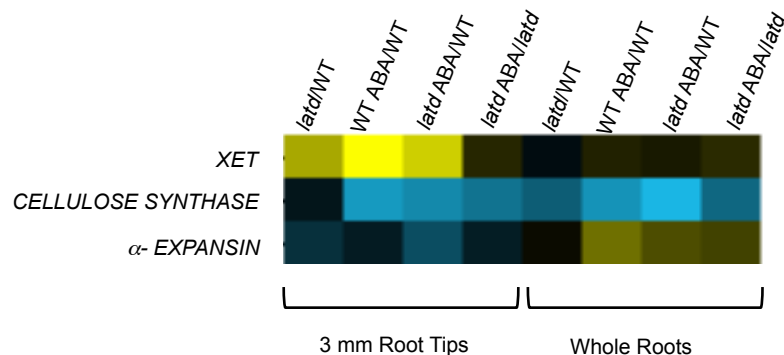
(C) Radial patterning is normal in *latd* mutant roots. Cross-sections of wild-type and *latd* mutant roots were taken at 1 cm away from the root tip. Sections were 90  $\mu\text{m}$  thick. Scale bars, 50  $\mu\text{m}$ .



**Figure 2. Relative expression of root meristem genes *MtWOX5* (A), *MtSTM* (B), *MtBBM1* (C) and *MtCYCLIN B-1,2* (D) in wild-type (WT) and *latd* mutant root tips.**

Root tips (3 mm) from seven-day old plants grown on BNM plates with or without 10  $\mu$ M ABA were harvested for qRT-PCR analysis. Graphs represent the average from three biological replicates, with each gene normalized to *MtUBC9*. Error bars represent standard error. Asterisks

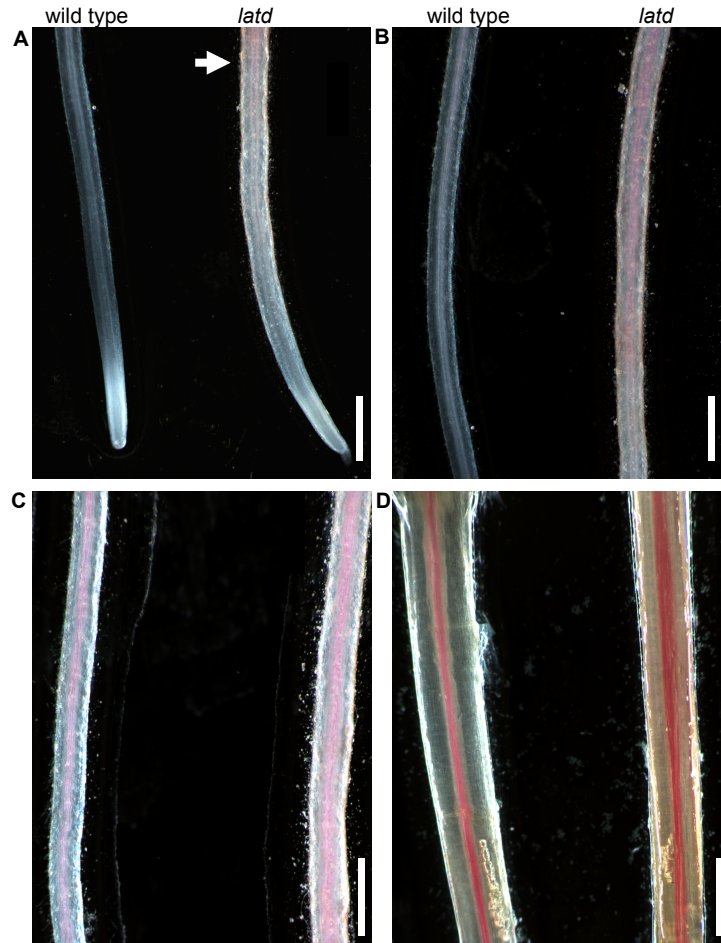
indicates a statistically significant difference between treatments in the same genotype, using one-way ANOVA test, with a p-value  $<0.05$ . qRT-PCR primers are listed in Table 1.



**Figure 3. The regulation of MtLATD/NIP and ABA on the expression of cell wall-associated genes in root tips and whole roots.**

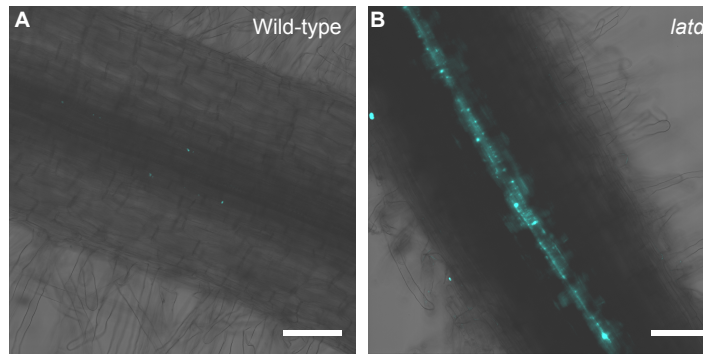
Heatmap displays the expression profile of MtXET, MtCELLULOSE SYNTHASE, and Mtα-EXPANSIN in *latd* root tips and whole root tissue, grown continuously with and without 10 μM ABA for 7 days. Different comparisons of gene expression are indicated on top of each lane of the heatmap.

Root tips (3 mm) or whole roots from seven-day old plants grown on BNM plates with or without 10 μM ABA from three biological replicates were harvested for qRT-PCR analysis. Relative gene expression was normalized to two housekeeping genes, MtUBC9 and MtPDF2. Yellow color indicates up-regulated, blue color indicates down-regulated, and black color indicates no change between the comparisons. qRT-PCR primers are listed in Table 2.



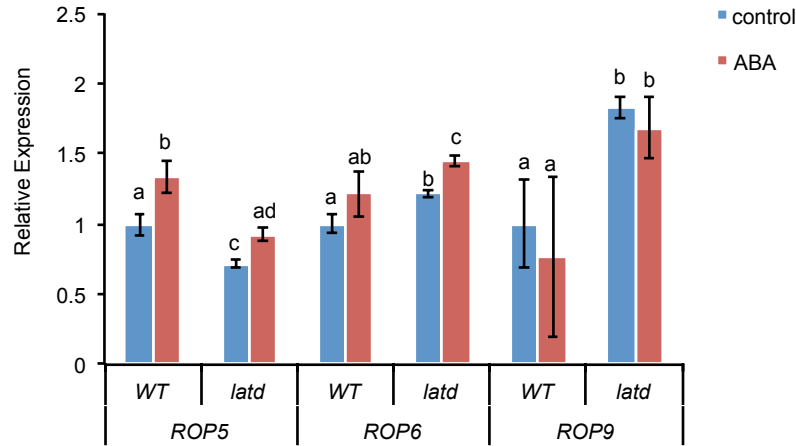
**Figure 4. *latd* mutants have ectopic lignin accumulation in roots and hypocotyls.**

7-day old wild-type and *latd* whole roots (A-C) and hypocotyls (D) were stained with phloroglucinol-HCl for lignin. Lignin staining is in red. *latd* mutant roots have ectopic lignin accumulation closer to the root tips than that in wild type (A, arrow), and this trend can be seen along upper parts of the roots (B-C), as well as in the hypocotyl in *latd* mutants. Images are representative for staining of 4-5 plants for each genotype. Scale bars, 1 mm.



**Figure 5. Young *latd* mutant roots have increased callose deposition.**

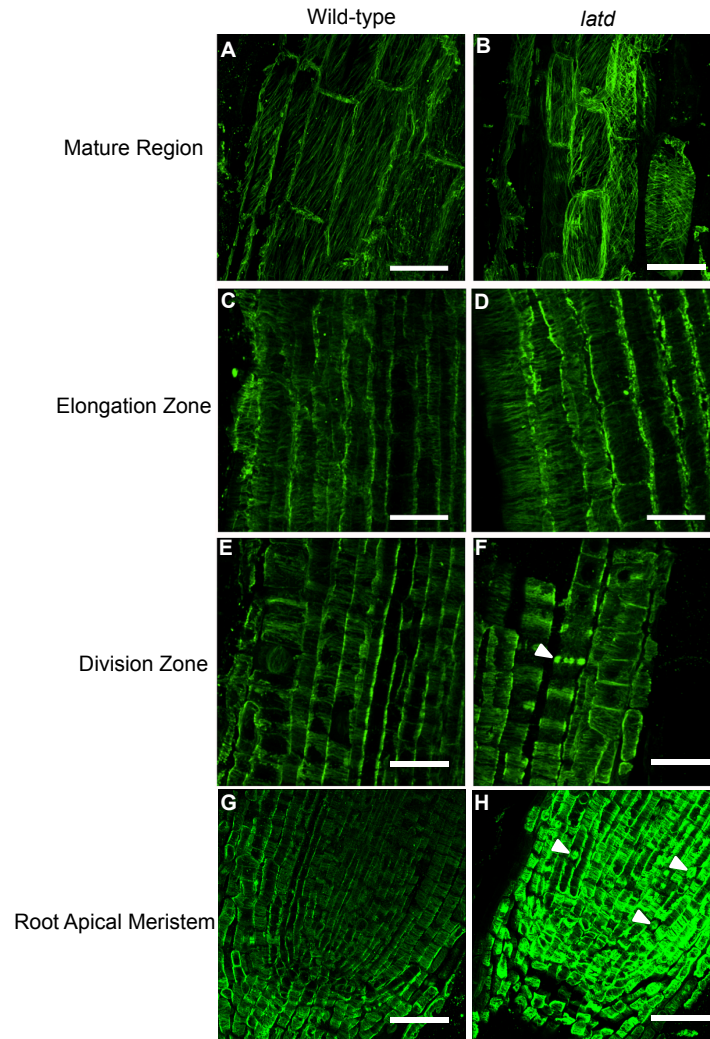
Confocal images of seven-day old wild-type (A) and *latd* roots (B) were stained with aniline blue for callose. Images shown here were taken from the mature region of the roots, using UV excitation, 420 nm emission filter, and overlaid with DIC. Images are representative for staining of 4-5 plants for each genotype.



**Figure 6. Relative expression of MtROP5, MtROP6 and MtROP9 in wild-type (WT) and *latd* mutant roots.**

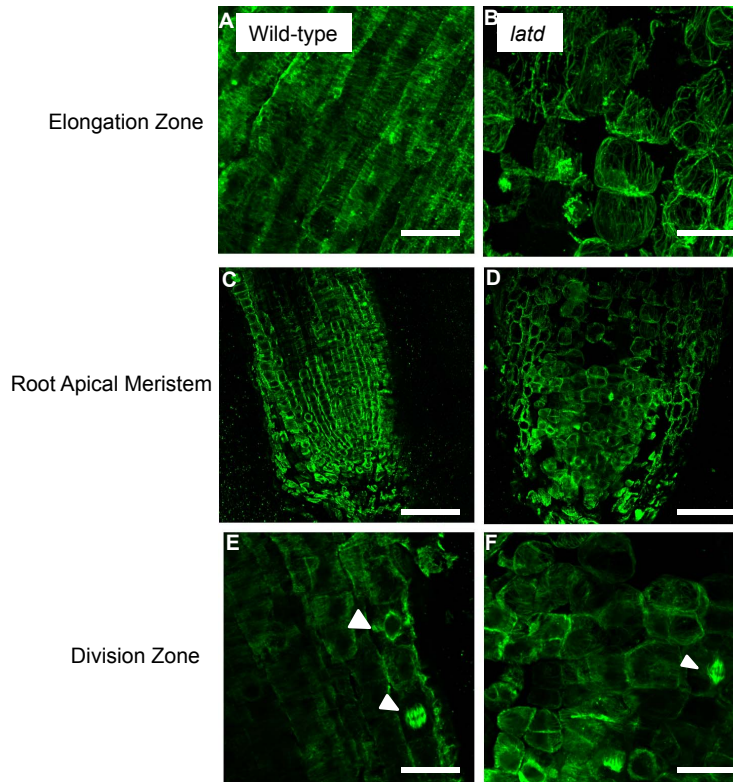
Seven-day old plants grown on BNM plates were transferred to medium with or without 10  $\mu$ M ABA for 24 hours. Whole roots were harvested for qRT-PCR analysis. Graphs represent the average from three biological replicates, with each gene normalized to MtUBC9 and MtPDF2. Error bars represent standard error. Letters indicates a statistically significant difference between treatments in the same genotype within the same gene, using one-way ANOVA test, with a p-value <0.05. qRT-PCR primers are listed in Table 2.





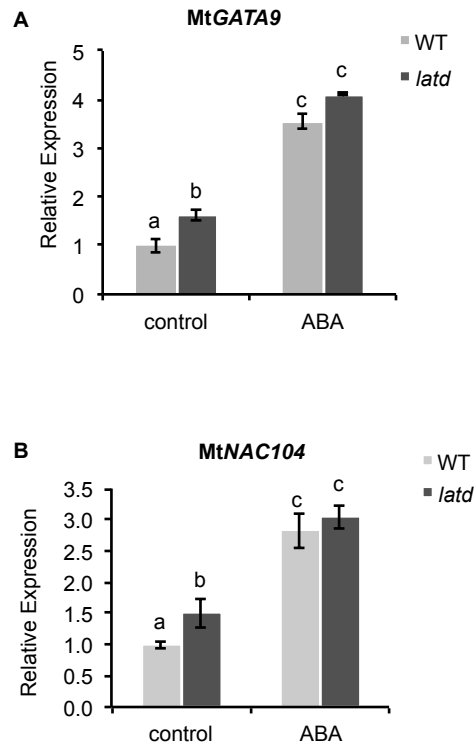
**Figure 7. Microtubule organizations in different developmental zones of 4-day old wild-type and *latd* mutant roots.**

Immunolabeling of microtubules in the mature region (A, B), elongation zone (C, D), division zone (E, F) and root apical meristem (G, H) of 4-day old wild-type and *latd* mutants. Note the orientation of microtubules in the mature region is parallel to the growth direction (A and B), transverse to the growth direction (C and D) in the elongation zone. Cell division is observed in *latd* mutant root division zone (arrowheads in F and H). Scale bars, 25  $\mu$ m.



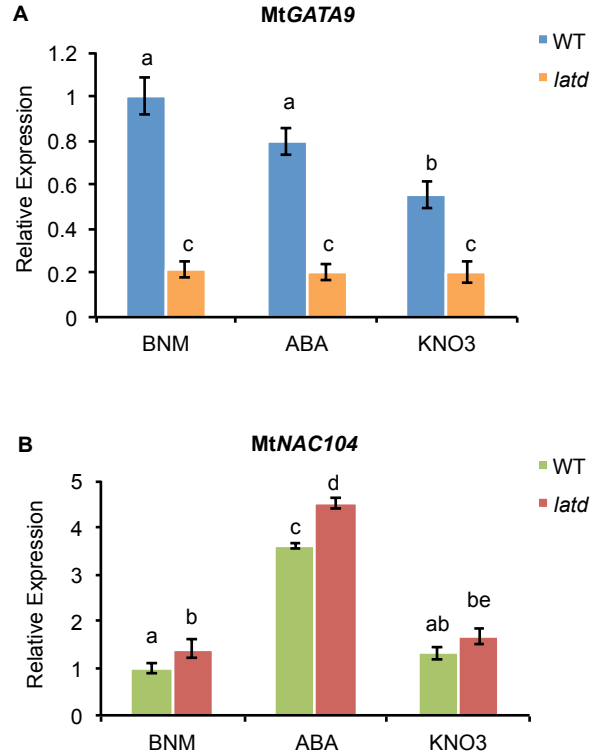
**Figure 8. Microtubule organizations in different developmental zones of 21-day old wild-type and *latd* mutant roots.**

Immunolabeling of microtubules in the elongation zone (A, B), root apical meristem (C, D) and division zone (E, F) of 21-day old wild-type and *latd* mutants. Note that the root apical meristem in *latd* mutant roots is disorganized (D), and microtubule organization in the elongation zone has changed from transverse to parallel to the direction of cell elongation (B). Cell division is still observed in *latd* mutant root division zone at this stage, despite of the fact that the RAM of *latd* mutants is disorganized (arrowheads in E and F). Scale bars, 25  $\mu$ m.



**Figure 9. The expression of *MtGATA9* and *MtNAC104* is regulated by *MtLATD/NIP* and ABA.**

Relative expression of *MtGATA9* (A) and *MtNAC104* (B) are both up-regulated in *latd* mutant roots, and can be induced by 10  $\mu$ M ABA, based on TF profiling data (Chapter 3). Four-day old plants grown on BNM plates with or without 10  $\mu$ M ABA continuously. Whole roots were harvested for qRT-PCR analysis. Graphs represent the average from three biological replicates. Error bars represent standard error. Letters indicates a statistically significant difference between treatments and genotypes, using one-way ANOVA test, with a p-value <0.05. qRT-PCR primers are listed in Table 3.



**Figure 10. ABA and nitrate regulation on the expression of *MtGATA9* and *MtNAC104*.**

Relative expression of *MtGATA9* (A) and *MtNAC104* (B) in wild-type and *latd* mutant roots treated with 10  $\mu$ M ABA and 10 mM KNO<sub>3</sub> for 3 days. Seven-day old plants grown on BNM plates were transferred to medium with 10  $\mu$ M ABA or 10 mM KNO<sub>3</sub> for 3 days. Whole roots were harvested for qRT-PCR analysis. Graphs represent the average from three biological replicates. Error bars represent standard error. Letters indicates a statistically significant difference between treatments and genotypes, using one-way ANOVA test, with a p-value <0.05. qRT-PCR primers are listed in Table 3.

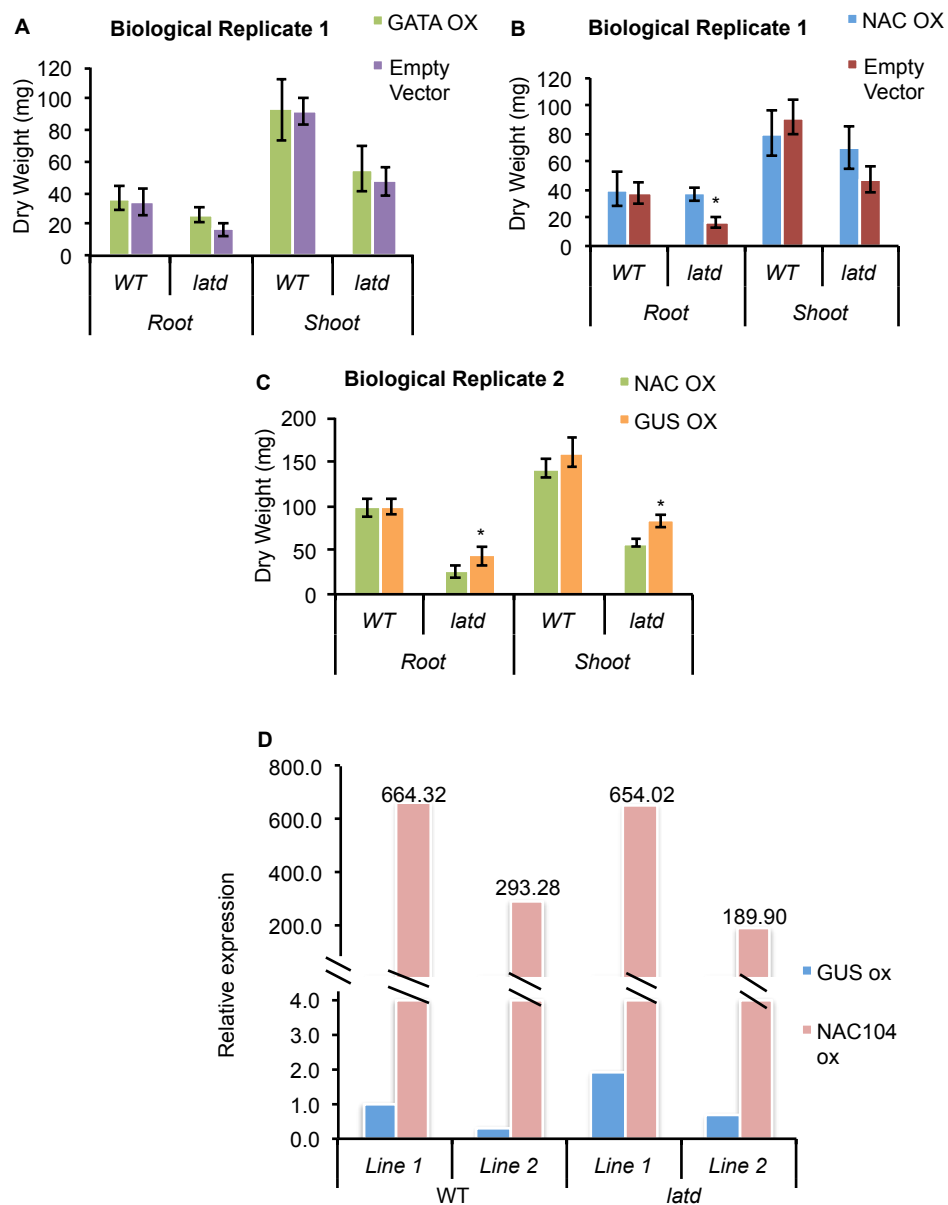
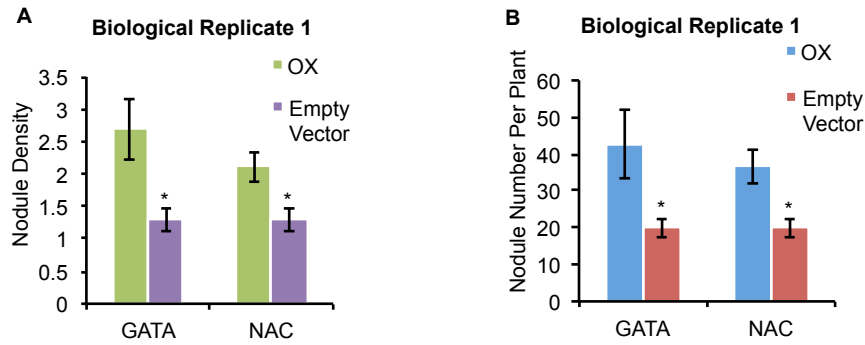


Figure 11. . Tissue dry weight of *M. truncatula* wild-type (WT) and *latd* mutant hairy roots transformed with MtGATA9 or MtNAC104 overexpression vectors (OX), compared to empty vector in biological replicate 1 (A-B) and to GUS overexpression in biological replicate 2 (C). All composite plants were transferred to surface and sand (v/v, 1:1) 3 weeks after transformation, and grown in greenhouse for 4 weeks until harvested. Graphs represent average of root or shoot dry

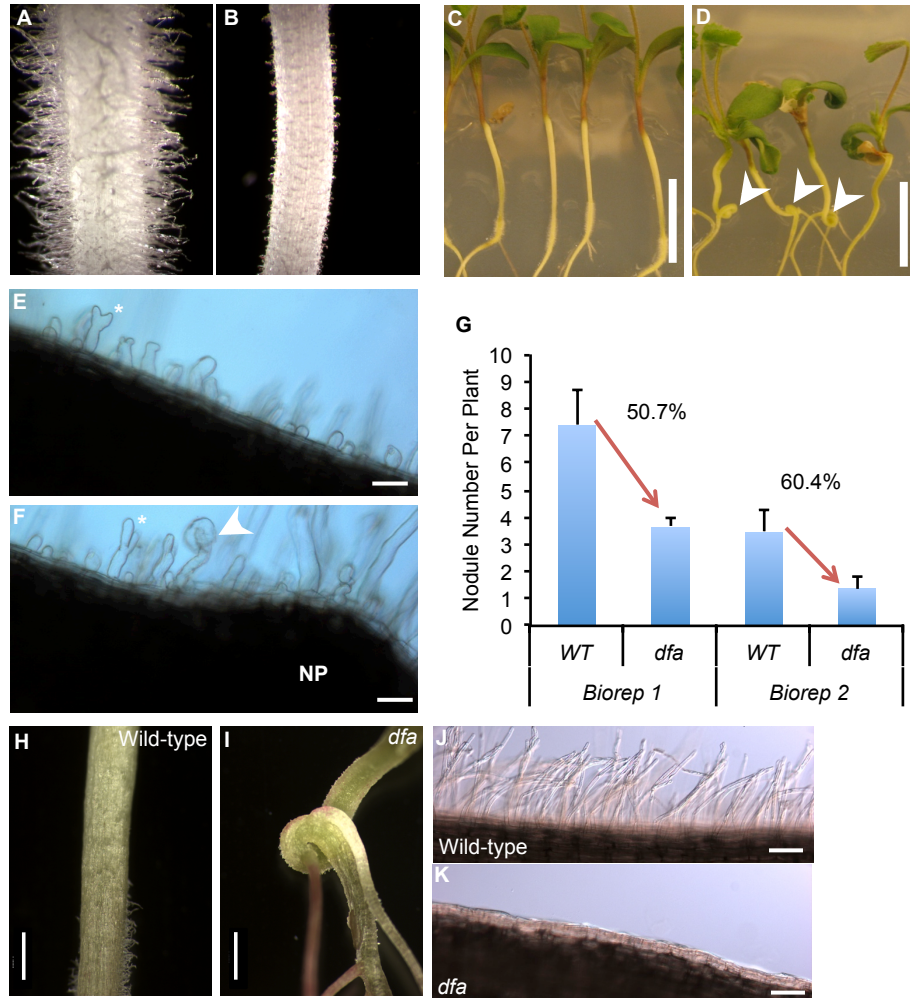
weight, with n=5 to 8 independent lines for biological replicate 1, and n=8 to 12 independent transformed lines for biological replicate 2. Error bars represent standard error. Asterisks indicate a statistically significant difference between plants transformed with overexpression and empty vector within the same gene, using one-way ANOVA test with a p-value<0.05.

(D) *MtNAC104* overexpression was confirmed in two independent transformation lines of wild-type and *latd* mutant roots. Number above bars indicate relative expression values of each line.



**Figure 12. Nodulation phenotype of *M. truncatula* wild-type (WT) hairy roots transformed with MtGATA9 or MtNAC104 overexpression vectors (OX).**

Graphs display the average nodule density (A) and nodule number per plant (B) in MtGATA9 or MtNAC104 overexpression lines, compared to root transformed with empty vector in biological replicate 1. All composite plants were transferred to turf and sand (v/v, 1:1) 3 weeks after transformation, and grown in greenhouse. All plants were inoculated at 5 day post transfer with *S. meliloti* 1021 and nodules were counted 14 day post inoculation. Graphs represent the average from n=5 to 12 independent lines for biological replicate 1. Error bars represent standard error. Asterisks indicate a statistically significant difference between plants transformed with overexpression and empty vector within the same gene, using one-way ANOVA test with a p-value <0.05.



**Figure 13. Mutant phenotypes of *M. truncatula* root hair mutant *dfa*.**

(A-B) Root hair mutant *dfa* has shorter root hairs (B) compared to wild-type (A). Images were taken from the mature region of the roots in 7-day old plants grown on BNM plates.

(C-D) *dfa* mutants often exhibit curly hypocotyl phenotypes, compare to wild-type plants, which have straight hypocotyls. Images were taken from 7-day old plants grown on BNM plates. Note the curls formed on the hypocotyls of *dfa* (arrowheads in D). Scale bars, 1 mm.

(E-F) Root hair responses of root hair mutant *dfa* to inoculation with *S. meliloti* 1021. Root hair branching can be observed in *dfa* mutants 7-day post inoculation (asterisks in E and F), as well as



root hair curling (arrowhead in F). Nodule primordia (NP) is also formed at this stage in mutant *dfa* (F). Scale bars, 50  $\mu$ m.

(G) Nodule number per plant in root hair mutant *dfa*, compared to wild-type plants at 14-day post inoculation. A reduction of nodule number is observed in mutant *dfa* from two biological replicates (Biorep 1 and Biorep 2). The percentage reduction of nodule number in mutant *dfa* compared to wild-type plants is indicated for each replicate. Plants were grown in liquid BNM medium in growth pouches and inoculated with *S. meliloti* 1021 at Day 5. Nodule numbers were counted at 14-day post inoculation, with n=8 and 11 in replicate 1 for wild-type and *dfa* respectively, 9 and 11 in replicate 2 for wild-type and *dfa* respectively. Error bars indicate standard error.

(H-K) Curly hypocotyl phenotype (H and I) and the more severe root hair phenotype (J and K) in the progeny of a plant from the F3 population from the backcross. Scale bars, 1 mm in H and I, 50  $\mu$ m in J and K.